Conformational changes and *in vitro* core-formation modifications induced by site-directed mutagenesis of the specific N-terminus of pea seed ferritin

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Plant ferritin has a three-dimensional structure predicted to be very similar to that of animal ferritin. It has, however, an additional specific sequence of 24 amino acids at its N-terminus named extension peptide (EP). In order to determine precisely the interactions between EP and other domains of the pea seed ferritin subunit, three point mutations were performed. The mutated residues were chosen by three-dimensional computer modelling of the pea seed ferritin subunit structure [Lobréaux, Yewdall, Briat and Harrison (1992) Biochem. J. **228**, 931–939]. The mutant recombinant proteins were expressed in *Escherichia coli* and purified to homogeneity; all the mutants were found to be assembled as 24-mers. When Ala-13 was replaced by His, as in mammalian ferritins, ferroxidase activity was significantly reduced. Moreover, *in vitro* iron-core formation in Pro-X → Ala, Lys-R → Glu and Ala-13 → His mutants was increased after denaturation by urea followed by renaturation; this was also observed with the EP deletion mutant (rΔTP/EP). The recombinant ferritins were also analysed using tryptophan fluorescence spectra. The rΔTP/EP, Pro-X → Ala and Lys-R → Glu mutants were found to be more susceptible to denaturation by urea than the native rΔTP pea seed ferritin.

INTRODUCTION

Iron storage by ferritin is a widely distributed mechanism in living organisms for coping with the insolubility and toxicity of iron in the presence of oxygen [1]. Ferritin is a multimeric spherical protein assembled from 24 subunits which define a shell surrounding a central cavity which is able to accommodate up to 4500 iron atoms [1,2]. Two distinct types of ferritin, BFR and FTN, have been characterized in *Escherichia coli* [3,4]. Although in mammals only one type of ferritin molecule has been found, it does consist of two different subunits, H and L, which are present in various ratios in the same molecule depending on the tissue in which it is located [2,5]. L- and H-subunits share 47% amino acid sequence identity, and L-rich ferritins are associated with long-term iron storage whereas H-rich molecules are more concerned with iron detoxification [6]. This difference has been investigated by structure/function studies using recombinant homopolymers and various related variants engineered by site-directed mutagenesis. The superiority of the L-chain in ferrihydrite nucleation can be explained by the presence of additional carboxy groups (Glu-49, -57, -60) on their cavity surface [7,8]. Rapid oxidation of FeII by H-ferritin is due to a ferroxidase centre composed of the seven conserved residues Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141 [2,9–11].

In plants, despite reports of microheterogeneity within ferritin sequences of the same species [12,13], only one type of ferritin subunit with amino acid sequence identity with animal ferritins ranging from 39 to 49% has so far been characterized [2]. Although it contains a conserved ferroxidase centre [2,13], plant ferritin subunit can be considered an H/L-hybrid because of the presence of additional glutamate residues (Glu-57 and Glu-60) reminiscent of those found on the cavity side of B-helices of animal L-type ferritin [2,14]. Ferritin purified from pea seed and a recombinant pea seed ferritin purified from *E. coli* have a higher ferroxidase activity than recombinant human L-ferritin, but slightly lower than recombinant human H-ferritin [13,15]. Important differences between animal and plant ferritins have been outlined on the basis of sequence comparison and computer-model prediction of pea seed ferritin subunit structure [14]. First, the channels found at the 4-fold symmetry axes of the molecule are predicted to be hydrophilic in the pea seed ferritin quaternary structure whereas they are hydrophobic in animal ferritin. Second, an additional plant-specific sequence is observed at the N-terminus [16]. The first part of this specific N-terminus encodes a transit peptide responsible for the plastid targeting of plant ferritin; it is found in plant ferritin precursors synthesized in the cytoplasm of plant cells, and is cleaved on uptake of ferritin subunit by cholorplasts [17–19]. This transit peptide must be deleted in order to produce assembled functional recombinant pea seed ferritin in *E. coli* [13]. The second domain of the N-terminal region specific to plant ferritin belongs to the mature wild-type ferritin subunit and has been named extension peptide (EP) [16]. Even though its deletion does not prevent expression of functional recombinant pea seed ferritin in *E. coli*, it plays a role in protein stability [13] and is cleaved in response to iron release *in vitro* [20] and during the early steps of germination [21]. Conformational changes have been suggested to occur in recombinant pea seed ferritin lacking EP which could be responsible for the increase in iron-core formation on renaturation of urea-denatured recombinant ferritin despite a loss in stability [13].

Here we investigate the effects of deletion of EP and of three point mutations on the conformation of native, denatured and renatured recombinant proteins as observed by tryptophan

Abbreviation used: EP, extension peptide.

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fluorescence. In addition, the effects of these mutations on *in vitro* iron-core formation by native and renatured proteins are also reported.

MATERIALS AND METHODS

Engineering of site-directed mutants

In a previous study DNA fragments coding for pea seed ferritin lacking the transit peptide (ΔTP) or both the transit and the extension peptides (ΔTP/EP) were obtained by PCR using PeSd2 pea seed ferritin cDNA as template [13]. The oligonucleotides used in the PCR amplification were designed to introduce *Nde*I and *Bam*HI restriction sites at each end of the resulting PCR fragments. These PCR fragments were cloned by conventional methods [22] into the pET3a *E. coli* expression vector [23]. This orientated cloning put the transcription of DNA inserts under the control of the T7 promoter in the BL21 (DE3) *E. coli* strain.

The ΔTP DNA fragment was cloned into pBluescript KS phagemid vector (Stratagene). This phagemid was used to synthesize single-stranded uracil-containing DNA in the CJ236 *E. coli* strain, for subsequent site-directed mutagenesis as described by Kunkel et al. [24]. Three different single amino acid mutations were performed (see under Rationale and Figure 1): Lys-R replaced by Glu (KRE), Pro-X replaced by Ala (PXA) and Ala-13 replaced by His (A13H). The different oligonucleotides used to introduce these independent mutations into the ΔTP single-stranded uracil-containing template were respectively: 5′-GAAGAGGTTAAGGAGGATTATCTTGC-3′, 5′-TATCTTGCTGTTGCTTCTGTTCCTCTTG-3′ and 5′-CGTCAGAATTTTCATGATGAATGTGAATC-3′. After second-strand synthesis and elimination of the non-mutated uracil strand into the MV1190 *E. coli* strain, pBKS phagemids containing the ΔTP DNA fragments harbouring the desired mutations were analysed by double-strand sequencing by the method of Sanger et al. [25]. Both strands of the DNA inserts of each selected ferritin mutant, as well as pΔTP and pΔTP/EP, were fully sequenced in order to confirm that no undesirable mutations had been introduced during the course of either PCR amplification or *in vitro* site-directed mutagenesis. Point mutated inserts (KRE, PXA, A13H) were introduced into the *Nde*I and *Bam*HI restriction sites of the pET3a expression vector and transformed into the BL21 (DE3) *E. coli* strain in order to express mutated recombinant ferritins.

Expression and purification of recombinant pea seed ferritins

Expression of recombinant pea seed ferritins in *E. coli* was adapted from the method of Blackwell and Horgan [26], allowing the production of soluble recombinant proteins by decreasing the formation of inclusion bodies. A preculture of the BL21 (DE3) *E. coli* strain transformed by the relevant plasmids described above was performed at 37 °C in Luria–Bertani broth supplemented with 1 M sorbitol and 2.5 mM betaine hydrochloride until an A_{600} of 0.6–1 was reached. The temperature of the culture was then lowered to 25 °C and 0.4 mM isopropyl β-D-thiogalactoside was added to induce expression of the recombinant ferritins. Expression was maintained at 25 °C for 3 h until cells were harvested by centrifugation.

Cell pellets were then resuspended in 20 mM Tris/maleate buffer, pH 8.0, lysed by sonication for 4 min and centrifuged for 5 min at 12100 *g* (10000 rev./min) in a JA-20 Beckman rotor to eliminate cellular debris. The supernatant containing the expressed soluble recombinant ferritin was collected and incubated at 75 °C for 10 min to precipitate the bulk of the protein. After centrifugation for 5 min at 12100 *g* in a JA-20 Beckman rotor, the supernatant was loaded on a gel-filtration AcA22 column (IBF, Villeneuve la Garenne, France) previously calibrated with purified pea seed ferritin. Proteins contained in the fractions corresponding to the elution profile of pea seed ferritin were then loaded onto a Mono Q anion-exchange column (Pharmacia). The recombinant ferritin was eluted through a 0–0.5 M NaCl gradient in 20 mM Tris/maleate buffer, pH 8.0. Iron concentration was measured in each fraction. The presence of pure ferritin in iron-rich fractions was assessed by SDS/PAGE. Recombinant ferritin was eluted with 0.27 M NaCl under these conditions. These fractions were pooled and then concentrated to 3 mg of protein/ml using Centricon devices (Amicon), before storage at 4 °C in 20 mM Tris/maleate buffer, pH 8.0, containing 10% glycerol.

Protein determination and analysis

Protein concentrations were determined by the method of Bradford [27]. Electrophoresis was performed as previously described under denaturing conditions [21] and in non-denaturing 4–25% polyacrylamide gradient gels in TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA) [20]. Gels were stained with Coomassie Brilliant Blue R250.

Iron content of purified ferritin was determined as previously described [20].

The kinetics of iron uptake by various ferritins were studied as described by Santambrogio et al. [28] using a molar apoferritin/iron ratio of 1:1000. Freshly prepared iron(II) sulphate was added to a final concentration of 0.1 mM to 0.1 μmol (≈ 50 μg) of various recombinant apoferritins in 100 mM Hepes buffer, pH 6.8, at room temperature. Formation of an amber colour resulting from iron(III) incorporation into ferritins was monitored by measuring the increase in A_{310}.

Iron-uptake measurements with renatured protein

Each recombinant ferritin (50 μg) was denatured overnight at 4 °C in 6 M urea. The volume of the denaturing reaction mixture was less than 50 μl. Denatured ferritins were renatured by increasing the volume to 900 μl with 20 mM Tris/maleate buffer, pH 8.0, and by adding 100 μl of 1 M Hepes buffer, pH 6.8. After a 2 h renaturation period at room temperature, the kinetics of iron uptake were studied by adding freshly prepared iron(II) sulphate (0.1 mM). Formation of the resulting amber colour of iron(III) incorporated into ferritins was monitored as described above.

Tryptophan fluorescence measurements

Fluorescence spectra were recorded with an SLM Aminco spectrofluorimeter with excitation and emission windows both of 8 nm. The excitation maximum was at 296 nm for native pea seed ferritins. Emission spectra were recorded at an excitation wavelength of 296 nm.

Spectra of the native proteins were obtained using 20 μg of recombinant ferritin in 1.6 ml of 20 mM Tris/maleate buffer, pH 8.0. Spectra of the denatured proteins were obtained after denaturation of 20 μg of ferritin overnight at 4 °C in 6 M urea or in 6 M guanidinium chloride. The volume of the denaturing reaction mixture was less than 50 μl. It was then increased to 1.6 ml with 20 mM Tris/maleate buffer, pH 8.0, and the spectra were rapidly recorded. Spectra were obtained for renatured proteins after denaturation as described above, followed by dilution of the denatured ferritins with 20 mM Tris/maleate buffer, pH 8.0, to a final volume of 1.6 ml. The spectra were recorded after incubation for 2 h at room temperature. Back-

ground fluorescence caused by urea or guanidinium chloride was subtracted from each spectrum.

RESULTS

Rationale

It has been suggested that the EP found specifically at the N-terminus of plant ferritin may play a role in protein stability [13,20]. In order to determine precisely its function, we produced recombinant ferritins in *E. coli* that were altered in this particular sequence, and characterized them by tryptophan fluorescence and their ability to form iron cores *in vitro* under different states of denaturation. We performed some of the experiments on ferritin molecules that have previously been denatured and renatured because such treatment increased the iron-storage capacity of recombinant pea seed ferritin that lacks its EP, when compared with the wild-type [13]. Furthermore, as stated by Dill and Shortle in a recent review [29], studies on non-native and denatured states of proteins are important to understand protein

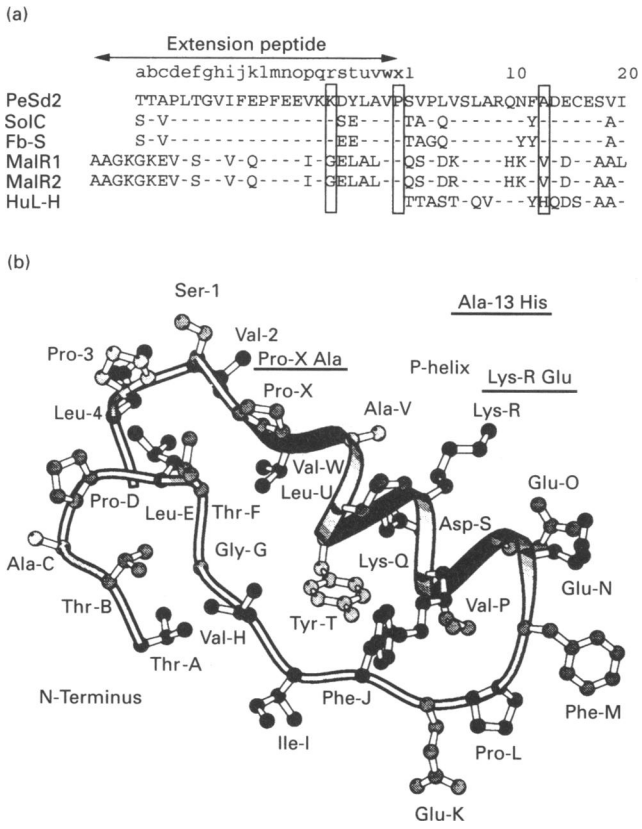

Figure 1 Structure of the specific N-terminus sequence (EP) of plant ferritin subunit

(**a**) Comparison of the EP amino acid sequence from the different plant ferritin subunits so far characterized: pea seed ferritin (PeSd2) [13,14]; maize ferritin from roots loaded with iron (MalR1 and MalR2) [12]; soyabean ferritin from iron-loaded cultured cells (SolC) [19] and french bean ferritin from seedlings (Fb-S) [31]. HuL-H is the N-terminal sequence of the ferritin H-subunit found in human liver [2]. Lower-case letters indicate the EP sequence [14], and numbers 1–20 correspond to the numbering of amino acids of human ferritin H-subunit [2]. Boxed amino acids indicate the positions chosen for site-directed mutagenesis; the third box (position 13) corresponds to His-13 in the human ferritin-H subunit sequence. (**b**) Computer-generated model of the three-dimensional structure of the pea seed ferritin EP sequence. Variants produced by site-directed mutagenesis are underlined (adapted from ref. [14]).

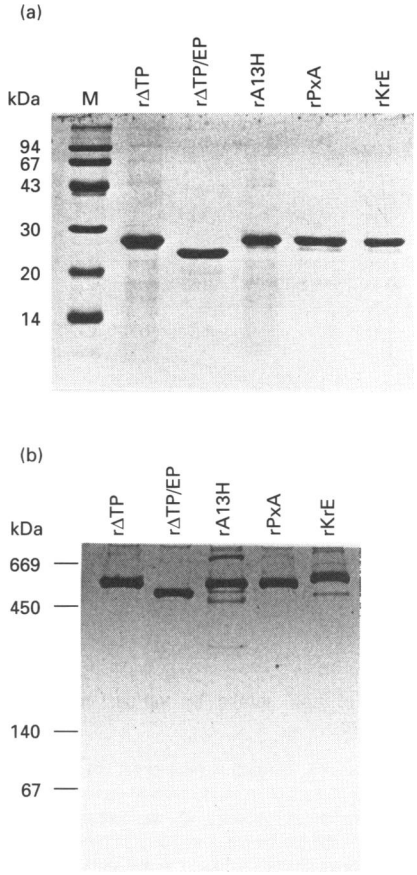

Figure 2 Characterization of purified recombinant pea seed ferritins by gel electrophoresis

(**a**) Under denaturing conditions, 5 μg of each purified recombinant pea seed ferritin was loaded on an SDS/15% polyacrylamide gel. (**b**) Under native conditions, electrophoresis was performed on a 4–25% polyacrylamide gradient gel (8 cm × 6.5 cm × 0.1 cm) in TBE buffer at a constant 10 mA for 15 h; 10 μg of each purified recombinant pea seed ferritin was loaded on the gel. Gels were stained with Coomassie Blue R250. M, molecular-mass markers.

folding and stability, as stability is defined as the difference in free energies between native and denatured states.

Deletion of the EP sequence was an obvious, but drastic, choice in order to investigate the role of this domain in structure/function relationships of plant ferritin. We also studied three site-directed mutants chosen on the basis of the following observations. The EP sequence of plant ferritin subunit is partly conserved in the various sequences so far reported (Figure 1a). An additional α-helix of 11 residues (three turns) has been predicted to form in the EP of pea seed ferritin and has been named the P-helix (Figure 1b) [14]. Interestingly, the two proline residues flanking the P-helix (Pro-L and Pro-X) are conserved in all plant ferritin EP sequences (Figure 1a). Asp-14 which replaces Gln in human H-ferritin is also conserved in all known plant ferritin sequences (Figure 1a); a hydrogen bond from $O^{\delta 1}$ of Asp-14 to the main chain carbonyl O of Pro-X provides a link to the EP sequence. The first-directed mutant engineered was therefore Pro-X → Ala (Figure 1b), which should alter both the P-helix and the link between the EP sequence and downstream domains of the subunit conserved between plant and animal ferritin. A further link between the EP sequence and downstream domains has also been indicated by the prediction [14] that

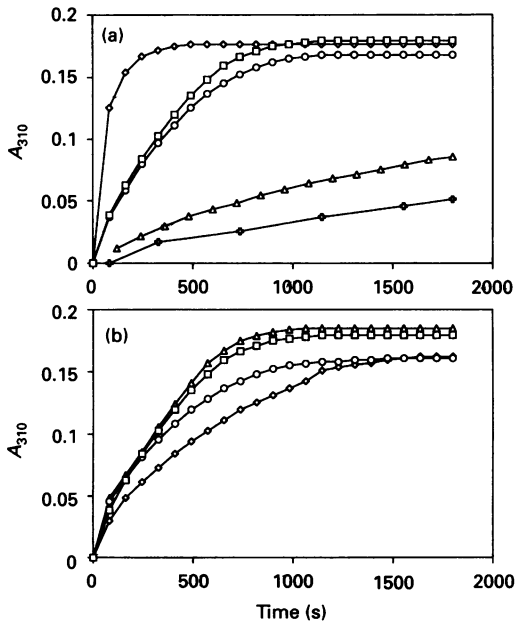

Figure 3 Kinetics of iron uptake by various recombinant apoferritin homopolymers *in vitro*

(**a**) Progress curves of iron-core formation in recombinant pea seed apoferritin (□), in its corresponding EP deletion mutant (○), in recombinant human H-apoferritin (◇) and in recombinant human L-apoferritin (△). A control (✣) was performed by replacing recombinant apoferritins with BSA. (**b**) Progress curves of iron-core formation in recombinant pea seed apoferritin (□), and in the rA13H (◇), rPXA (○) and rKRE (△) variants. Fe(II) atoms (1000 per molecule) were added as $FeSO_4$ to the proteins in Hepes buffer, pH 6.8.

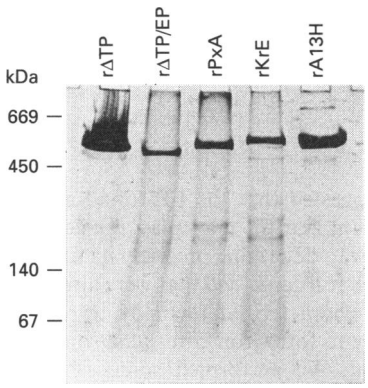

Figure 4 Analysis of denatured recombinant pea seed ferritins by non-denaturing PAGE

Recombinant pea seed ferritins were denatured by 6 M urea overnight at 4 °C at pH 7.0. Electrophoresis conditions were the same as in Figure 2, except that the gels were run for 4 h.

replacement of His-13 by Ala or Val in ferritin from dicotyledonous or monocotyledonous plants respectively (Figure 1a) provides a space that allows Lys-R from the P-helix to interact with $O^{\delta 1}$ of Asn-124. It should be noted that Lys-R is conserved between the ferritin EP sequence of dicotyledonous plants (pea, soyabean and French bean in Figure 1a) but is replaced by Gly in maize ferritin EP sequences (Figure 1a). The two other site-directed mutants engineered using pea seed ferritin were therefore Ala-13 → His, restoring the residue found in the human H-ferritin sequence, and LysR → Glu (Figures 1a and 1b); both mutations should affect the interaction between the residue found at position R of the P-helix with Asn-124.

Characterization of purified recombinant pea seed ferritins

Expression of the recombinant pea seed ferritins rΔTP, rΔTP/EP, rKRE, rPXA and rA13H in *E. coli* at 25 °C in the presence of sorbitol and betaine gave soluble proteins that were purified as previously described [13]. The purity of these proteins was assessed by SDS/PAGE (Figure 2a). The subunit of these various proteins has a constant molecular mass, except for rΔTP/EP which has a lower molecular mass because of the deletion of the 24 amino acids of the EP sequence. Analysis of these proteins on non-denaturing polyacrylamide gradient gels indicates that they are all assembled as high-molecular-mass molecules (Figure 2b). Except for two, they migrate at the level of the rΔTP protein which has previously been shown to migrate with the purified pea seed ferritin [13]. The rΔTP/EP protein has a lower molecular mass because of deletion of the first 24 amino acids from the N-terminus, and the rKRE has a slightly higher apparent molecular mass, indicating a possible conformational alteration which will be discussed below. These recombinant 24-mer pea seed ferritins were all found to contain between 50 and 70 iron atoms per molecule (results not shown).

These proteins have been analysed, by measurement at 310 nm of amber-colour formation, for their capacity to form iron cores *in vitro* (Figure 3). As previously described rΔTP and rΔTP/EP form their mineral cores more slowly than recombinant human H-ferritin (rHF) (Figure 3a), even though these proteins contain a consensus ferroxidase centre [13]. The kinetics of iron-core formation of the variants rKRE and rPXA are similar to those observed for the wild-type (rΔTP). The rA13H variant, however, has decreased ferroxidase activity, which is particularly noticeable during the first 500 s of the reaction (Figure 3b).

In vitro core formation of recombinant pea seed ferritins on renaturation after denaturation by urea

We have shown previously that the iron-core-formation capacity of rΔTP/EP is significantly increased, compared with rΔTP [13], after denaturation by urea followed by renaturation by dilution as described by Santambrogio et al. [28]. We wanted to know therefore whether point mutations KRE, PXA and A13H, which are predicted to alter the structure of pea seed ferritin (see under Rationale and ref. [14]), have any effect on this characteristic. After overnight denaturation in 6 M urea, electrophoretic analysis of these proteins showed that they were still assembled into multimeric forms (Figure 4), indicating a 'compact denatured' state as defined by Dill and Shortle [29] (see the Discussion section). On renaturation, the wild-type recombinant pea seed ferritin RΔTP has the same iron-storage capacity as native rΔTP, although its ferroxidase activity is reduced (Figure 5). As previously reported [13], rΔTP/EP has an iron-storage capacity on renaturation that is clearly higher than that of renatured rΔTP (Figure 5). Although the ferroxidase activity of the variant rA13H was shown to be lower than the other recombinant ferritins (Figure 3b), its iron-storage capacity on renaturation was also increased by the same factor as that observed for the variant lacking the EP sequence (Figure 5). Interestingly, the variants rPXA and rKRE have a considerably increased storage capacity on renaturation which is calculated to be 100% more than that of native rΔTP/EP and rΔTP (Figures 3a and 5); note that the progress curve of iron-core formation of the variant

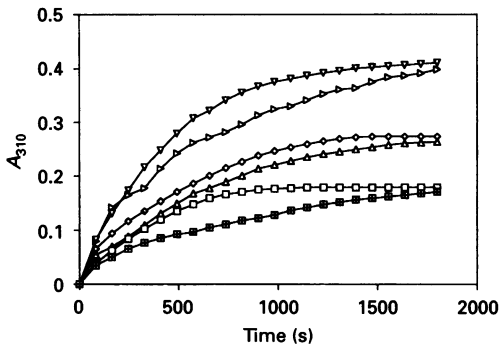

Figure 5 Kinetics of iron-core formation by various recombinant pea seed apoferritins after denaturation/renaturation

Purified recombinant pea seed ferritins were denatured by 6 M urea and renatured by dilution as described by Santambrogio et al. [28]. Fe(II) atoms (1000 per molecule) were added as $FeSO_4$ to the proteins in Hepes buffer, pH 6.8. Iron-core formation was measured by recording A_{310}. □, rΔTP, ⊞, rΔTP (denatured and renatured); ◇, rΔTP/EP (denatured and renatured); △, rA13H (denatured and renatured); ▽, rPXA (denatured and renatured); ▷, rKRE (denatured and renatured).

rKRE has a complex sinusoidal aspect which has been reproducibly observed.

It can be concluded that mutagenesis of the amino acids predicted to be involved in the interactions of the EP sequence with other domains of pea seed ferritin produced differences in the capacity of these recombinant proteins for iron-core formation after renaturation of 'compact denaturation' states of the protein. It was therefore important to evaluate the possible conformational changes that could have been introduced by these mutations.

Differential denaturation by urea of the various recombinant pea seed ferritins

In order to evaluate the conformational changes in the structure of recombinant pea seed ferritin resulting from EP deletion and point mutations, tryptophan fluorescence spectroscopy was performed on the various protein mutants. In their native form, all the proteins excited at 296 nm were found to have a fluorescence maximum near 330 nm (Figure 6). Two types of fluorescence-emission behaviour were subsequently recorded. First, the spectra of urea-denatured rΔTP and rA13H were almost unchanged compared with their respective native forms, although the fluorescence maximum was slightly quenched at red wavelengths. Second, a significant decrease in the fluorescence emission maxima of urea-denatured rΔTP/EP, rKRE and rPXA proteins was observed (Figure 6). After renaturation the fluorescence emission maxima returned almost to the values observed for the native proteins, but complete reversion to the native spectra was not observed (Figure 6). When denaturation was performed with 6 M guanidinium chloride at pH 7 instead of 6 M urea, for the same time and at the same temperature, all the recombinant pea seed ferritins exhibited the same emission fluorescence spectrum, which was characterized by a strongly decreased maximum and a shift to the red wavelengths when compared with the native forms (Figure 6). The proteins denatured by this method were not observed as multimeric forms on non-denaturing PAGE (results not shown), indicating that this treatment probably caused disassembly.

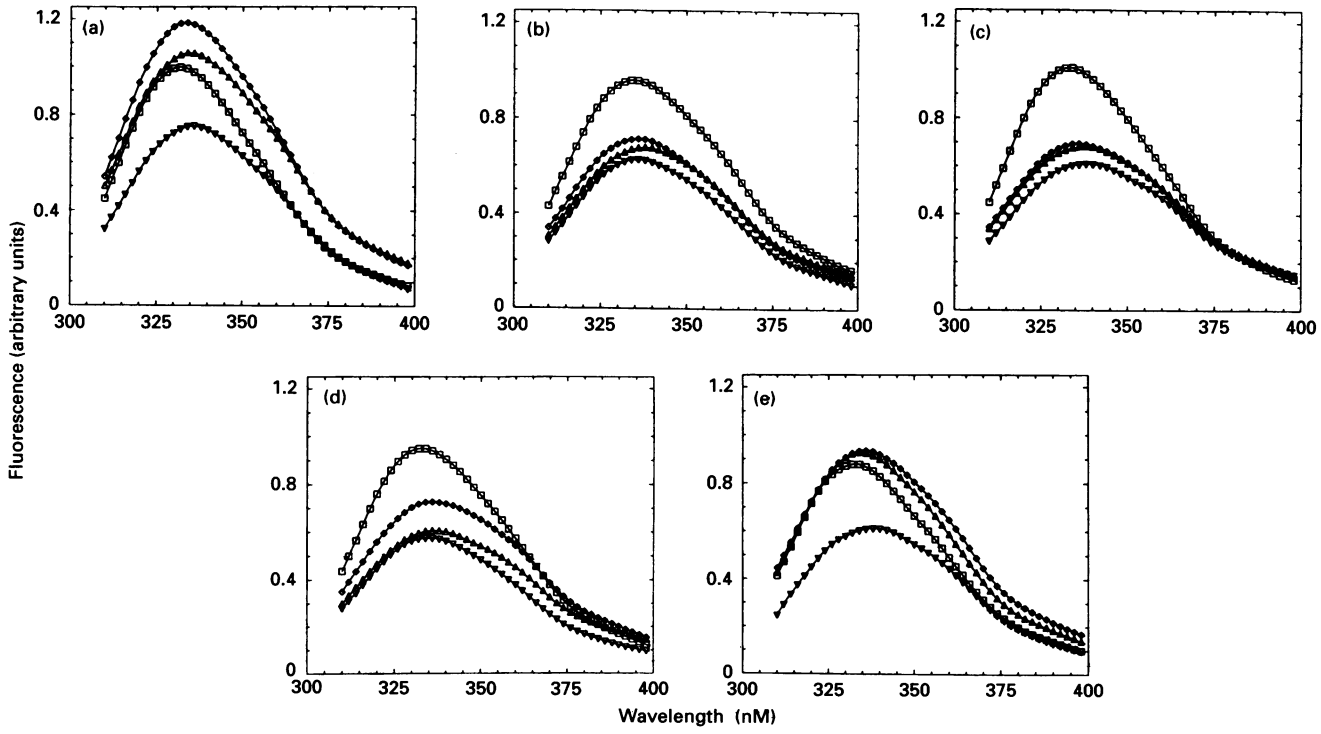

Figure 6 Analysis of native, denatured and renatured recombinant pea seed ferritins by fluorescence emission spectroscopy

Denaturation and renaturation were performed as described in the Materials and methods section. Each spectrum was obtained using 20 μg of each recombinant protein in 20 mM Tris/maleate buffer, pH 8.0. Excitation was at 296 nm. (**a**) rΔTP; (**b**) rΔTP/EP; (**c**) rPXA; (**d**) rKRE; (**e**) rA13H. ◇, urea-denatured and renatured protein; ▽, proteins denatured with guanidinium chloride; △, proteins denatured with urea; □, native proteins.

DISCUSSION

The plant-specific sequence found at the N-terminus of the plant ferritin subunit (EP) has been suggested to be an important structural determinant of the stability of the protein because it is cleaved during iron release *in vitro* [20] and during the germination process *in vivo* [21]. The use of recombinant technology has enabled demonstration that deletion of the EP sequence does not prevent assembly of a functional protein, the deletion mutant having almost the same ferroxidase activity as the wild-type recombinant ferritin (Figures 2 and 3 and ref. [13]); although the mutant was found to be less soluble, it had an increased iron-storage capacity on renaturation after denaturation by 6 M urea (Figure 5 and ref. [13]). These observations can be explained by conformational changes resulting from disruption of interactions between various amino acids of the EP sequence and downstream sequences (see under Rationale). Indeed, the tryptophan fluorescence emission spectra of the various mutants analysed here, in their native form are almost identical and very similar to the spectra recorded for the recombinant wild-type pea seed ferritin (Figure 6). Complete disassembly of these proteins can be obtained by using the strong denaturing agent guanidinium chloride, as for human ferritins [28,30]; this treatment yielded identical spectra for the various recombinant proteins, with decreased maxima and a slight shift to the red wavelengths when compared with spectra of the native forms (Figure 6). Therefore EP deletion and the point mutants PXA, A13H and KRE are not distinguishable, in either their native or disassembled forms, by means of tryptophan emission fluorescence spectroscopy. It is therefore not surprising to observe that rΔTP/EP, rPXA and rKRE proteins have almost the same iron-core-formation kinetics *in vitro* when in their native form as wild-type rΔTP protein. However, it is important to note that the rA13H mutant behaves differently with a decreased ferroxidase activity when in its native form (Figure 2). Therefore the presence of Ala-13 in plant ferritin instead of His-13 in animal ferritin has a direct effect on the ferroxidase activity of the pea seed ferritin, which is independent of the predicted effect this change may have on the interaction of Lys-R with Asn-124 (see under Rationale).

The various recombinant pea seed ferritins denatured by 6 M urea are still assembled into multimeric forms, but the urea treatment allows discrimination between different denaturation states characteristic of each variant, which can be observed by tryptophan fluorescence emission spectra. Such an observation is in agreement with the fact that the denatured state of a protein results from the distribution of many microstates which change with the solution conditions and with the protein sequence [29]. The primary sequence of a protein is a major determinant of its denatured conformation, and it has been well documented that single amino acid changes can affect the denatured state (for review, see ref. [29]). In the present case, it is clear that 6 M urea treatment significantly changed the spectra of the rΔTP wild-type recombinant ferritin and the rA13H variant (Figure 6). Interestingly, urea-denatured rΔTP/EP, rPXA and rKRE proteins, although assembled into multimers as determined by native PAGE (Figure 4), produced tryptophan fluorescence emission spectra with lower maxima when compared with rΔTP and the rA13H mutant, demonstrating that these mutated ferritins have altered conformations, as revealed by urea denaturation. rΔTP and rA13H produced the same tryptophan fluorescence emission spectra after renaturation which were almost identical with those produced by their respective native and urea-denatured forms. In contrast, rΔTP/EP, rPXA and rKRE proteins produced tryptophan fluorescence emission spectra that were almost identical with those of their urea-denatured forms, i.e. different from their native forms. It can be concluded therefore that tryptophan fluorescence emission spectra of denatured and denatured/renatured recombinant pea seed ferritins enable observation of conformational changes induced by EP deletion and PXA and KRE point mutations. The denaturing mechanism of urea is probably weakening of hydrophobic interactions within a protein [32,33]. Therefore the conformational changes in plant ferritin variants revealed by urea treatment reported here could be related to differential modification of their internal hydrophobic interactions. These modifications correlate with functional changes, since rΔTP/EP, rPXA and rKRE proteins all have an increased iron-storage capacity on renaturation (Figure 5). However, although the different conformational states of each of the above-mentioned proteins cannot be discriminated by measurement of tryptophan fluorescence emission, it is likely that the changes differ in each variant because their iron-storage capacities are affected differently (Figure 5). Surprisingly, the increase in iron-storage capacity is much more significant for PXA and KRE than for rΔTP/EP in which the whole EP region is deleted. Although conformational changes induced by denaturation/renaturation of rΔTP and rA13H are not revealed by examination of the corresponding tryptophan fluorescence spectra (Figure 6), these proteins nevertheless have modified iron-storage properties; rA13H has an increased iron-storage capacity similar to that of rΔTP/EP, and the renatured rΔTP wild-type recombinant ferritin attains the same iron-storage capacity as its corresponding native form, but it does so at a reduced rate. Quantitative measurements of the different conformational changes resulting from urea treatment of the various apoferritin variants could be obtained by fluorescence-energy-transfer experiments.

In conclusion, deletion of the EP sequence and mutagenesis of amino acids predicted to be important in establishing interactions between EP and downstream sequences induced conformational changes in recombinant pea seed ferritins expressed in *E. coli*. However, these changes are not characteristic of the proteins analysed in their native forms, except for the Ala-13 → His variant which exhibited decreased ferroxidase activity in its native form. Conformational changes correlating with increased iron-storage capacity can only be observed after urea denaturation followed by renaturation.

We are indebted to Professor P. Arosio (Milano, Italy) for the gift of recombinant human H- and L-ferritin. We acknowledge Dr. R. Gibrat (INRA, Montpellier) for help with experiments based on tryptophan fluorescence emission, and Dr. H. Logan for careful reading of the manuscript. This work was supported by CNRS (URA 1178 and 573 and ATIPE grant no. 93N60/0563 to J.F.B.).

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Received 17 June 1994/8 September 1994; accepted 15 September 1994