Volume 336, number 2, 309–312 FEBS 13444 December 1993

Human ferritin H-chains can be obtained in non-assembled stable forms which have ferroxidase activity

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Received 22 October 1993; revised version received 11 Novemvber 1993

We found conditions to obtain the Leu-169→Arg mutant of human ferritin H chain in a stable and non-assembled state. The protein obtained is an oligomer of subunits with a high degree of structured conformation, and when concentrated it re-assembles into ferritin cages. Functional studies showed that (i) it promotes iron oxidation like the assembled ferritin, but at slower rate, (ii) it is readily precipitated by the oxidised iron unless apotransferrin or L-chain ferritin are added to sequester Fe(III). The results confirm that ferroxidase activity is located within the H-chain, and indicate that the cages of the fully assembled ferritins are important not only in maintaining iron in a soluble form, but also in eliciting the activity of the ferroxidase centres.

Ferroxidase; Ferritin; Iron protein; Protein assembly

1. INTRODUCTION

Ferritin is an almost spherical iron storage protein made from 24 subunits. Each subunit is folded into a 4-helical bundle conformation with a long loop connecting helices B and C. In most vertebrates the ferritins are composed of various proportions of two subunit types, H- and L-chains, genetically distinct but with about 50% sequence identity [1,2]. Studies of the recombinant homopolymers of human H- and L-type chains and of some variants indicated that in vitro the H-ferritin oxidises iron at rates several fold faster than the L-ferritin [3,4], and that L-ferritin appears to induce iron mineralization with higher efficiency than the H-ferritin [5]. Crystallographic studies identified a ferroxidase centre inside the protein shell of the H-chain involving two metal binding sites, A and B, only 3 Å apart [6,7]. The two sites involve residues Glu-27, Glu-61, Glu-62, His-65 and Glu-107 as ligands. Glu-27, Glu-62 and His-65 are substituted in the L-chain and it lacks ferroxidase activity [3,4], and so does the H-chain mutant in which Glu-62 and His-65 are artificially substituted [7].

The mechanism of ferritin iron uptake has not been completely clarified and it appears to involve an interplay between the ferroxidase centres and the cavity that accommodates the iron core [5]. Ferritin cages without ferroxidase centres were shown to take up iron, albeit slowly [8]. In contrast, single subunits or incomplete shells with ferroxidase centres have not been studied. Cavities are made by the assembly of 24 subunits, while the ferroxidase centres are expected to be made by the coming together of the H-chain ligands in the folding into the 4-helix bundle [6]. In principle, it is possible to have ferroxidase centres in folded but unassembled subunits. However, assembly interactions are strong, and disruption of ferritin quaternary structure usually yields unfolded peptides [9,10].

We have previously shown that assembly interactions of H ferritin can be weakened by site-directed mutagenesis without modifying ferroxidase activity [10]. Here we show that renaturation of the H-chain variant, Leu-169→Arg, yields stable subunit oligomers (here referred to as non-assembled ferritin) which retain a high degree of structured conformation. Functional studies show that (i) the subunits have ferroxidase activity, but lower than in the assembled cages, (ii) they are precipitated by the hydrolysis of the oxidised iron, unless iron binding proteins are added to the reaction mixture, (iii) L-chain homopolymers act as efficient Fe(III) binding proteins. It is concluded that the presence of fully assembled ferritin cages is important for the full activity of the ferroxidase centres.

2. MATERIALS AND METHODS

Production of the complete and correct sequences of human ferritin H- and L-chains from *E. coli* strains has been described [4,11]. The H-ferritin variant, Leu-169→Arg, is described in [10,12,13]. Expression, purification and apoferritin preparation were performed as de-

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Abbreviations: GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; CD, circular dichroism; rHF, recombinant human H-chain ferritin; rLF, recombinant human L-chain ferritin.

scribed in [5]. All ferritins were electrophoretically pure, and treated for iron removal before functional studies. Protein concentration was determined with BCA reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. When required, the samples were concentrated by a membrane ultra-filtration system (Amicon, Grace, Italy).

Far UV CD spectra were collected as described in [10]. Fluorescence spectra were collected with a Kontron SFM 25 spectrofluorimeter with an excitation window of 5 nm and an emission window of 10 nm. Apoferritins were denatured by incubation in 6 M GdnHCl, 0.1 M HEPES buffer, pH 3.5. Proteins were renatured by 10-fold dilution in 0.1 M HEPES buffer, pH 7.0, with 1 mM DTT. The conformational status of ferritin was derived from the ellipticity values at 222 nm and from fluorescence spectra with an excitation at 295 nm [14]. Fluorescence spectra were analysed for the excitation maxima and for the ratios of the emission at 354 nm and 327 nm, the maxima of the denatured and assembled states, respectively [14]. Gel-filtration experiments were performed on a Superose 12 FPLC column (Pharmacia, Biothec, Italy), equilibrated with 20 mM Tris, pH 7.4, 0.15 M NaCl.

Iron oxidation was started by adding the appropriate amount of Fe(II), as a freshly prepared 10 mM solution of ferrous ammonium sulphate in water, to iron-free assembled or non-assembled apoferritins in 0.1 M HEPES buffer, pH 7.0, to give a final concentration of 0.1 mM Fe(II). Iron oxidation was monitored either by the disappearance of Fe(II) with a discontinuous assay as described in Treffry et al. [15] or by the increase in optical reading at 310 nm [3]. Ferroxidase activity was also monitored using ovotransferrin (Diarconal, Recordati, Italy) as the acceptor of Fe(III) as described in [3,16].

3. RESULTS

3.1. *Renaturation of the H-chain mutant Leu-169→Arg*

We analysed the renaturation conditions of the H-chain mutant Leu-169→Arg, which is very much less stable than the H-chain wild-type [10,13]. The protein was denatured in 6 M GdnHCl, pH 3.5, and renatured by diluting it in buffer at pH 7.0 to a final concentration of 50–250 μg/ml [10]. Electrophoretic analyses showed that the renaturation product of Leu-169→Arg was a single species with a faster mobility than the 24-mer, while rHF re-assembled in ferritin cages (Fig. 1, lanes 1 and 2). Removal of the residual GdnHCl by dialysis had no effect on the renaturation product of Leu-169→Arg, but concentration to 1.5 mg/ml by membrane ultra filtration induced protein assembly with no significant formation of aggregates (Fig. 1, lane 3). We found that Leu-169→Arg assembled only at concentrations above 0.3 mg/ml, and that, once assembled, it behaved like the native protein with no evident dissociation down to concentrations of 20 μg/ml (not shown). The conformation of the non-assembled Leu-169→Arg was analysed. The tryptophan fluorescence spectra (Fig. 2) show that the emission maxima of the native (or re-assembled), non-assembled and denatured Leu-169→Arg were at 327, 333 and 354 nm, respectively (excitation at 295 nm). The ratios of the emissions at 354/327 nm were 0.64, 0.75 and 1.68, respectively, indicating that the non-assembled Leu-169→Arg is 90% in the folded conformation [10]. Far UV CD spectra had minima at 222 nm, with ellipticity values similar to those of the native proteins (not shown). Gel-filtration on a calibrated Superose 12 column indicated that the non-assembled Leu-169→Arg has an apparent molecular mass of about 65 kDa. These data indicated that the non-assembled Leu-169→Arg is a trimer or a dimer of structured subunits.

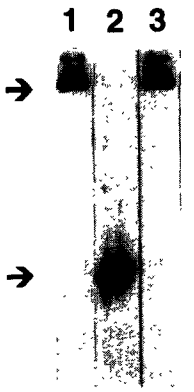

Fig. 1. Electrophoresis of ferritins (3 μg per well) in a 7.5% polyacrylamide gel, stained with Coomassie blue. Lanes: 1, renatured rHF at 0.2 mg/ml; 2, renatured H-chain mutant, Leu-169→Arg, at 0.2 mg/ml; 3, renatured Leu-169→Arg concentrated to 1.5 mg/ml. The arrows indicate the mobility of the assembled ferritin (top) and of the non-assembled subunit oligomers (bottom).

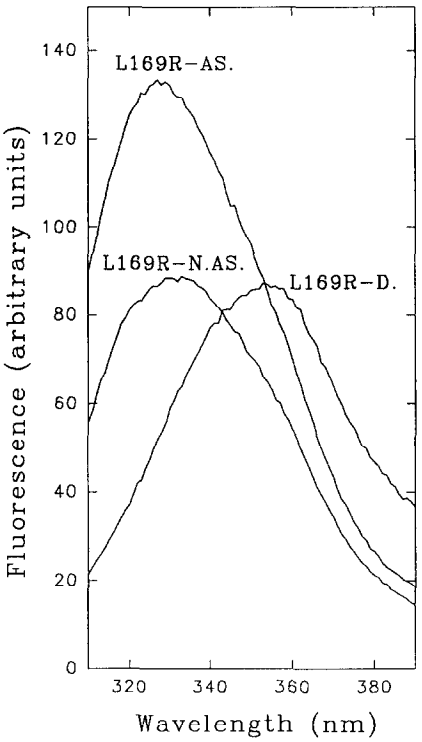

Fig. 2. Emission fluorescence spectra of H-chain mutant, Leu-169→Arg, in the assembled (AS), denatured (D) and non-assembled (N.AS.) states. Conditions: 50 μg/ml in 0.1 M HEPES buffer, pH 7.0, excitation at 295 nm.

3.2. *Functional studies of the non-assembled Leu-169→Arg*

Before functional studies, all the non-assembled Leu-

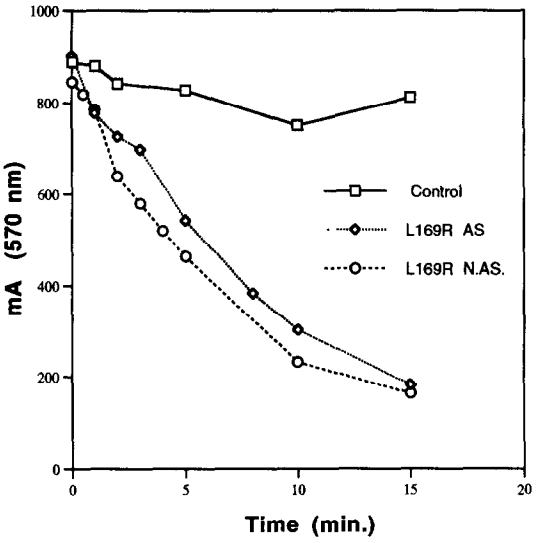

Fig. 3. Time-course of iron oxidation. Iron oxidation was followed by a discontinuous assay in which the concentration of Fe(II) was detected as Fe-ferrozine complex. Conditions: 0.1 M HEPES, pH 7.0, 0.1 mM ferrous ammonium sulphate, the control contained no protein, the assembled Leu-169→Arg (AS) was at 22 μg/ml, and the non-assembled Leu-169→Arg (N.AS.) was at 220 μg/ml.

169→Arg samples were analysed on gel electrophoresis to verify the absence of assembled species or aggregates. The activity was first analysed by monitoring the disappearance of Fe(II) by discontinuous assay for the formation of a Fe(II)–ferrozine complex [15]. The rate of iron oxidation induced by 220 μg/ml non-assembled Leu-169→Arg was significantly faster than the protein-free control and comparable to that of the 22 μg/ml assembled form (Fig. 3). The activities of both forms varied linearly with protein concentration (not shown), indicating that the specific activity of the non-assembled Leu-169→Arg was about 10% of that of the assembled form, in this reaction system.

In other experiments we evaluated the ferroxidase activity by monitoring the formation of diferric transferrin at 470 nm, as described in [3,16]. In this system ($\Delta_{A470\ nm/3\ min}$) the activity of the non-assembled Leu-169→Arg was about 25% of that of the assembled Leu-169→Arg (Fig. 4).

Next, we monitored iron oxidation as an increase in absorbance at 310 nm due to the formation of polymeric Fe(III) species (iron cores) [4]. The initial rate of the reaction ($\Delta_{A310\ nm/min}$) of the non-assembled Leu-169→Arg at 220 μg/ml was analogous to that of the assembled form at 22 μg/ml (Fig. 5), but turbidity developed after the first min of reaction with the non assembled protein, due to formation of protein–Fe(III) aggregates. Addition of 0.1 μM rLF prevented turbidity development, the progression plots reached the expected plateau (Fig. 5), and no precipitate was collected after the reaction. The initial rate of the reaction was the same in the absence and in the presence of rLF.

4. DISCUSSION

We show that the H-chain mutant, Leu-169→Arg, re-assembles less readily than H- and L-chains, and only at higher protein concentrations. The substitution of a Leu with an Arg in the strongly hydrophobic region of the four-fold axes weakens the inter chain interactions responsible for protein assembly and reduces protein stability [10]. More interesting was the finding that under conditions in which the mutant does not assemble, a single species is obtained: a dimer or a trimer of structured subunits. This is consistent with data showing that the subunit dimer is the most populated intermediate in ferritin renaturation [9] and that subunit dimers are in equilibrium with assembled bacterial ferritins [17].

The non-assembled Leu-169→Arg subunit oligomers promote iron oxidation at rates significantly faster than protein-free controls, indicating that they have both ferroxidase activity and native-like conformations. This is the first direct evidence that the ferroxidase centre is located within the subunit and it is in keeping with previous suggestions that ferritin subunit folding may occur independently of shell assembly [9]. The non-assembled subunits form insoluble aggregates during reaction with Fe(II) due to the hydrolysis of the oxidised iron. This may be expected from a ferritin without a cavity, and it was previously observed in a ferritin in which the cavity was altered by the deletion of the last 22 amino acids that form the hydrophobic channels [3]. Iron-induced protein aggregation, as monitored by turbidity development, was prevented by the addition of apo-ovotransferrin, which chelates Fe(III).

Also rLF added to Leu-169→Arg subunits prevented iron-induced protein aggregation, and it incorporated the oxidised iron. This appears an interesting model, and shows that ferroxidase centres in incomplete shells

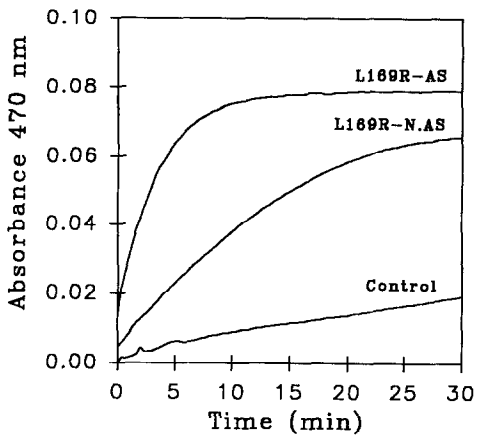

Fig. 4. Progress curves of the formation of Fe(III)–ovotransferrin complex. Conditions: 0.2 M sodium acetate, pH 6.0, 50 μM apo-ovotransferrin, 0.1 mM ferrous ammonium sulphate, 0.1 mg/ml of Leu-169→Arg in the assembled (AS) and non-assembled (N.AS) states. Control in the absence of ferritin.

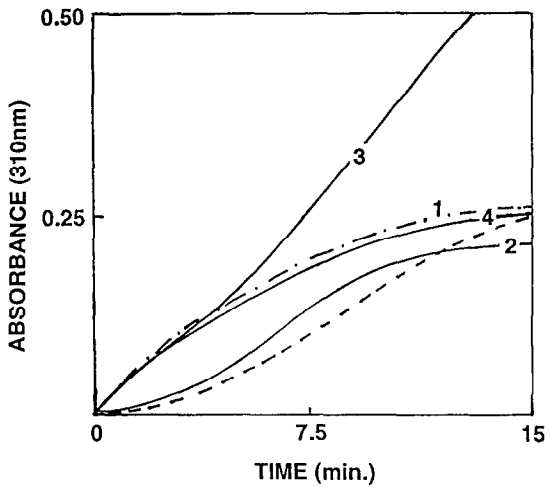

Fig. 5. Progression plots of ferritin iron core formation followed at 310 nm. Conditions: 0.1 M HEPES, pH 7.0, 0.1 mM ferrous ammonium sulphate, 30°C. Samples: (1) 22 μg/ml of assembled Leu-169→Arg; (2) 50 μg/ml of L-homopolymer; (3) 220 μg/ml of non-assembled Leu-169→Arg; (4) as in 3 with the addition of 50 μg/ml of rLF. The dashed line is the control in the absence of protein.

and ferroxidase-free ferritin cavities can cooperate (i.e. rLF), one inducing iron oxidation, and the other preventing protein precipitation, probably by inducing iron mineralization. This is in agreement with previous evidence that some of the iron oxidised by assembled rHF is taken up by rLF, and supports the hypothesis that rLF binds Fe(III) [5]. The ferroxidase centres appear to be about 10-fold more active in the assembled than in the non-assembled ferritins, a difference that may be due either to incorrect folding of the ferroxidase centre in the non-assembled subunits, or to cage effects. Iron probably enters the assembled ferritins via the hydrophilic channels where iron binding sites are located [6,18], and it mineralises on specific nucleation sites [8]. Site-directed modifications of both sites reduce the rate of iron oxidation to 15–50% of that of the wild-type [8,12]. It is conceivable that in the non-assembled subunits these binding sites are not formed or not functional, leading to a similar reduction in activity. This indicates that the ferritin cages are not simple sinks for iron, but that they affect iron oxidation, possibly by creating preferential pathways for iron delivery to and removal from the ferroxidase centres.

Acknowledgements: This work was supported by CNR Target Project Biotechnology and Bioinstrumentation and by CEC-BRIDGE grant n. BIOT-CT91-0262. We are grateful to Dr. E. Rovida for reading the manuscript.

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