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Mechanism of the self-assembly of apoferritin from horse spleen *

Cross-linking and spectroscopic analysis

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Abstract. Apoferritin from horse spleen can be reversibly dissociated at pH 2 or in 7.2 *M* G-HCl (pH 3.5). Reconstitution of the native icositetramer in 0.1 *M* TEA buffer (pH 7.9) in the presence of 1 m*M* EDTA and 3 m*M* dithioerythritol leads to yields higher than 80%. To monitor the kinetic mechanism, intrinsic fluorescence, far-UV circular dichroism, and covalent cross-linking with glutaraldehyde were applied.

The overall mechanism of assembly is characterized by a sequence of concentration-dependent association reactions involving "structured monomers" and a dimeric intermediate as the most prominent species, apart from trimers and dodecamers. The parallel decrease in monomers, dimers and trimers indicates that association equilibria precede the formation of the final assembly product.

The assembly reaction is accompanied by characteristic changes in fluorescence emission and dichroic absorption. To a first approximation, renaturation and reassociation may be quantitatively described by one single rate-determining second-order process, subsequent to fast folding steps at the monomer level.

Key words: Circular dichroism, cross-linking, ferritin, fluorescence, self-assembly

Introduction

Multimeric proteins acquire their functional state by the spontaneous and autonomous self-assembly of folded subunits. In vitro reconstitution after previous dissociation and denaturation has been applied to analyze the assembly mechanism, thus allowing one to correlate structure formation and biological function. The reaction may be assumed to reflect the mechanism of folding and association in vivo (Jaenicke 1987). The specificity of quaternary structure formation requires subunit association to be properly coordinated with the folding of the constituent polypeptide chains (Zettlmeissl et al. 1979; Rudolph et al. 1979).

The strategy in evaluating the mechanism of reconstitution involves the determination of rate-limiting folding and association reactions and the characterization of intermediates that are sufficiently populated to be accessible to physico-chemical analysis (Jaenicke and Rudolph 1986).

The present investigation is focussed on the self-assembly of apoferritin from horse spleen. The protein forms a hollow shell of external diameter 130 Å and internal diameter 75 Å, consisting of 24 structurally equivalent subunits in 432 symmetry. The cage-like structure of the protein provides two types of channels allowing the regulated storage of iron (Banyard et al. 1978; Clegg et al. 1980; Ford et al. 1984). Available high-resolution X-ray data clearly show that there is a considerable amount of intersubunit interactions within the symmetrical dimer and around molecular triads, while there is little interaction in the neighbourhood of the four-fold axis of the protein complex. Evidence from spectroscopic measurements proved that dissociation-reassociation equilibria of the protein show hysteresis at low pH (Wood and Crichton 1971; Crichton and Bryce 1973; Leach et al. 1976; Imai et al. 1981).

The self-assembly of its subunits is the most significant primary function of apoferritin in its nascent state. Previous attempts to resolve the mechanism of the reaction led to contradictory results.

* Dedicated to Professor Harold A. Scheraga on the occasion of his 65th birthday

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Abbreviations: CD, circular dichroism; DTE, dithioerythritol; G-HCl, guanidinium chloride; SDS, sodium dodecylsulfate; TEA, triethanolamine; Tris, *tris* hydroxymethylamino methane

Gel-filtration experiments suggested that assembly proceeds via dimers and tetramers, while partial polymerization and X-ray analysis pointed to dimers, trimers and hexamers as intermediates on the pathway of association (Crichton 1975; Stefanini et al. 1976, 1979; Banyard et al. 1978).

Making use of chemical cross-linking and fluorescence emission spectroscopy, the following experiments give evidence for the dimer and trimer as intermediates on the assembly pathway.

Materials and methods

Substances. Ferritin from horse spleen was purchased from Boehringer, Mannheim, dithioerythritol (DTE) from Roth (Karlsruhe), glutaraldehyde (purissimum, 25% (w/v) aqueous solution) and agarose (high electroendosmosis) from Fluka (Basel), acrylamide, bis-(acrylamide), ammonium persulfate, sodium dodecylsulfate (SDS), N,N,N′,N′-tetramethylethylenediamine from Serva (Heidelberg), guanidinium chloride (G-HCl) (ultrapure) from Schwarz-Mann (Orangeburg, N.Y.) and Sepharose 6B-Cl from Pharmacia (Uppsala). All other reagents were A-grade substances from Merck (Darmstadt). Quartz twice-distilled water was used throughout.

Standard buffer was 0.1 *M* triethanolamine-HCl (TEA) pH 7.9, containing 1 m*M* EDTA and 3 m*M* DTE.

Preparation of apoferritin. Apoferritin was prepared from ferritin by reduction with 1% (v/v) thioglycolic acid in 0.1 *M* glycine buffer containing 30 m*M* EDTA. The pH was adjusted to pH 4.5 using 1 *N* NaOH. To remove excess thioglycolate, the protein was dialyzed with the same buffer, and subsequently with 0.1 *M* TEA pH 7.6 in the presence of 1 m*M* EDTA and 1 m*M* DTE. To remove dimers and higher polymers of the apoferritin complex (10%–30%, depending on the sample), Sepharose 6B-Cl chromatography (3.5× 80 cm) in TEA buffer pH 7.6 was applied. Fractions containing the 450 kDa protein were concentrated by ultrafiltration (Amicon diaflo with PM 10 membranes). The concentration of the stock solution was determined by protein absorbance making use of $A^{1\%}_{280\,\mathrm{nm},\,1\,\mathrm{cm}} = 8.82$ (Bryce and Crichton 1973).

Denaturation and dissociation of apoferritin was accomplished by 10 min incubation in 7.2 *M* G-HCl (0.1 *M* phosphate buffer pH 3.5 plus 1 m*M* EDTA and 1 m*M* DTE, 20 °C).

For *renaturation and reassociation,* the denaturation mixtures were diluted with 0.1 *M* TEA buffer pH 7.9 plus 1 m*M* EDTA and 3 m*M* DTE at 20 °C; final protein concentration 2–45 μg/ml, final G-HCl concentration 0.15 *M.*

The *kinetics of reassociation* were monitored by intrinsic protein fluorescence, circular dichroism and chemical cross-linking.

Fluorescence spectra were measured in a Hitachi-Perkin Elmer MPF 44A spectrophotometer, equipped with a corrected spectra accessory: $\lambda_{exc} = 280$ nm, $\lambda_{em} = 300 - 360$ nm. Reassociation can be estimated from the intrinsic protein fluorescence at $\lambda_{em} = 350$ nm.

Circular dichroism measurements made use of a JASCO J-500A CD spectrophotometer, equipped with a DP-500N data processor. The degree of reconstitution was calculated relative to the final dichroic absorption determined after 24 h.

Cross-linking. Particle distributions in the time course of reconstitution were analyzed by glutaraldehyde fixation. Aliquots taken at defined times were reacted with 2.5% (w/v) glutaraldehyde for 1.5 min at 20 °C. For details see Hermann et al. 1981, 1983; Zettlmeissl et al. 1982; Jaenicke and Rudolph 1986.

SDS polyacrylamide gel-electrophoresis was performed on agarose-stabilized gradient gels with 2.4%–21.5% acrylamide and a 1.15%–0.06% agarose counter gradient. *Gel-buffer:* 0.38 *Tris*-HCl pH 8.8, 0.5% SDS (final concentrations); *reservoir buffer:* 0.025 *M Tris*-HCl, 0.19 *M* glycine, 0.1% SDS. Electrophoresis was performed at 800–1,000 V.h.

Staining of the protein bands was achieved by a modified silver staining method according to Merril et al. (1984). Scanning of the gels was performed at 560 nm using a Quick Scan densitometer (Desaga, Heidelberg) and an Ultroscan laser densitometer (LKB, Uppsala). The given cross-linking procedure was optimized with respect to glutaraldehyde concentration and incubation time; since the conditions for quantitative fixation may be different for the various intermediates on the assembly path, the cross-linking conditions for the reassociating protein were analyzed in detail (see Results). The degree of cross-linking (> 85%) remained unchanged in the range of protein concentrations under study. Assemblies not covalently linked were dissociated by 15 min incubation at 100 °C in the presence of 7.5% (w/v) SDS plus 50 m*M* DTE.

Results

Optimization of the cross-linking reaction

Chemical cross-linking by glutaraldehyde has been successfully applied to establish equilibrium distri-

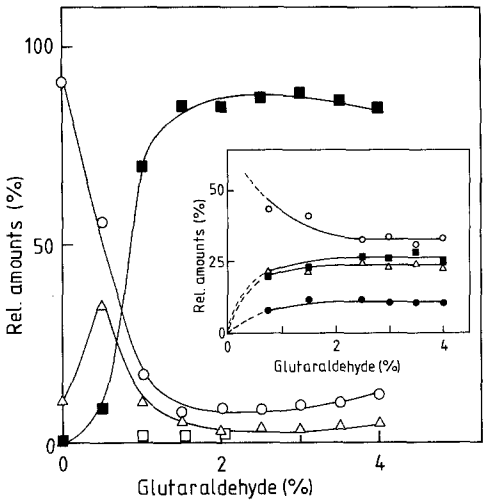

Fig. 1. Cross-linking of native apoferritin from horse spleen (22.6 µg/ml) by 1.5 min incubation at varying glutaraldehyde concentration; standard buffer at 20 °C. The relative amount of monomers (○), dimers (△), dodecamers (□), and icositetramers (■) was determined by densitometry after SDS-polyacrylamide/agarose gel-electrophoresis. *Insert:* Influence of glutaraldehyde concentration on the particle distribution *during reassociation.* Reconstituting apoferritin (44.8 µg/ml) was cross-linked (1.5 min, 20 °C) by adding glutaraldehyde 0.5 min after initiation of reconstitution in standard buffer. Monomers (○), dimers (△), trimers (●), icositetramers (■)

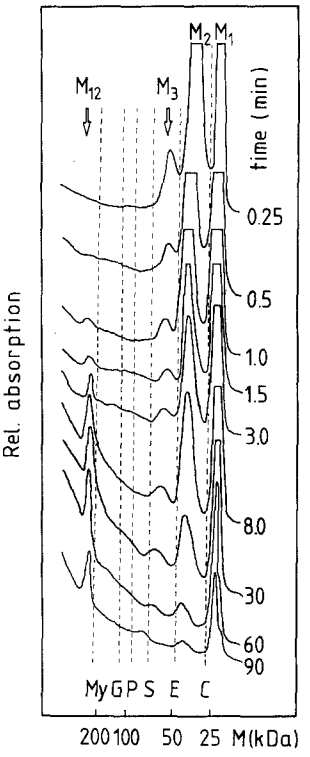

Fig. 2. Kinetics of the reassembly of apoferritin at 22.6 µg/ml in standard buffer at 20 °C. Cross-linking (1.5 min, 20 °C) at given times in the presence of 2.5% (w/v) glutaraldehyde. Densitometric analysis of the cross-linked *intermediates* after SDS polyacrylamide/agarose gel-electrophoresis. Because of the unbalanced relative ratios, the scanning profiles do not include the icositetramer. *Abbreviations:* M_i, assembly intermediates with i indicating the number of subunits. Vertical *dashed lines* indicate the reference proteins: *My*, myosin; *G*, β-galactosidase; *P*, phosphorylase b; *S*, bovine serumalbumin; *E*, egg albumin; *C*, carbonic anhydrase

butions as well as kinetic intermediates on the assembly pathway of oligomeric proteins (Jaenicke and Rudolph 1986). In order to avoid artifacts, the method must fulfil three requirements: *(i)* the cross-linking reaction must be fast compared to the association reaction under consideration, *(ii)* the cross-linking reagent must not form wrong interparticle cross-bridges, and *(iii)* the cross-linking conditions must not affect the reconstitution mechanism. To satisfy these requirements for a given system, the cross-linking conditions have to be optimized.

In a first series of experiments, cross-linking of native apoferritin was performed. At >1.5% (w/v) glutaraldehyde, optimum cross-linking yields were obtained after 1.5 min incubation time. Under these conditions >85% cross-linking was achieved (Fig. 1). Increasing the glutaraldehyde concentration or the reaction time did not change the results. The residual 15% of incompletely cross-linked protein is dissociated by 15 min incubation in 7.5% (w/v) SDS solution in the presence of 50 m*M* DTE at 100 °C, yielding monomer, dimer and dodecamer bands after SDS gel-electrophoresis; they may be explained by the competition of intra- and intersubunit cross-linking (Jaenicke and Rudolph 1986).

Fixation of the native protein does not necessarily imply that intermediates of association (which may be less stable than the native protein) are cross-linked as well. Therefore, the cross-linking conditions had to be confirmed for the reassociating system. "Saturation" of the cross-linking reaction was monitored by incubating reconstituting protein at high concentrations(45 µg/ml) after 0.5 min reactivation with varying amounts of glutaraldehyde. At a glutaraldehyde concentration ≧2% (w/v), the relative yields of monomers, dimers, trimers and icositetramers were found to remain constant (Fig. 1, insert). Thus, optimum cross-linking conditions to monitor the reassembly of apoferritin were: 1.5 min incubation at 20 °C in the presence of 2.5% (w/v) glutaraldehyde, protein concentration up to 45 µg/ml.

Cross-linking analysis of the kinetics of reassociation

The kinetics of the self-assembly of apoferritin using cross-linking and subsequent SDS-polyacrylamide/agarose gel-electrophoresis result in a complex association pattern. The reassembly at 22.6 µg/ml protein concentration is illustrated in Fig. 2. It starts from

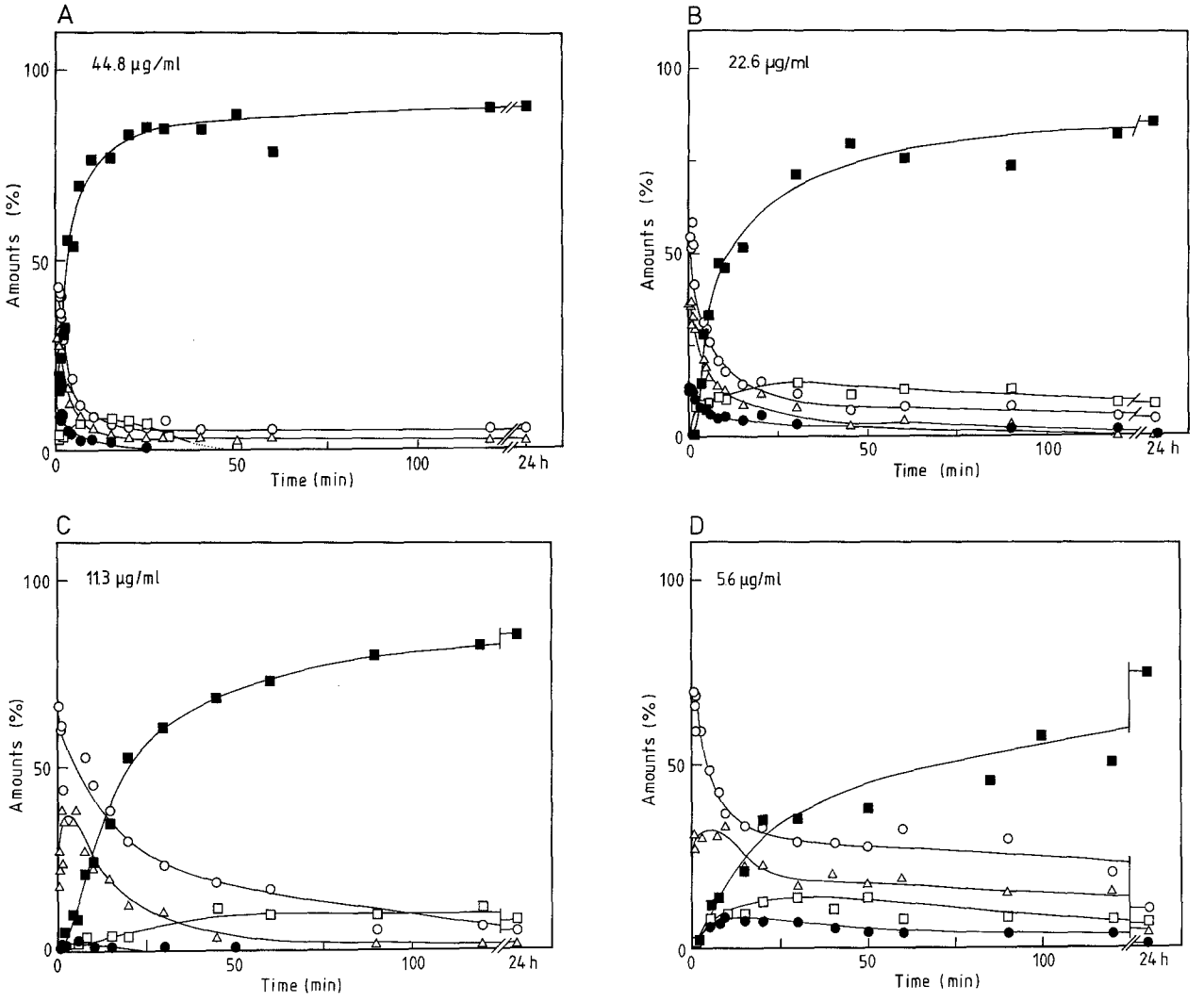

Fig. 3A – D. Reconstitution of apoferritin from horse spleen as determined by cross-linking and subsequent SDS polyacrylamide/agarose gel-electrophoresis. The kinetics of reassociation were determined from the peak areas of the densitometric analysis. Reconstitution at 20 °C by dilution in standard buffer after 10 min incubation (20 °C) in 7.2 *M* G-HCl pH 3.5. Reassembly at the following protein concentrations (µg/ml): 44.8 (**A**); 22.6 (**B**); 11.3 (**C**); 5.6 (**D**). Cross-linking as described in Fig. 2, symbols as in Fig. 1

the homogeneous monomer and yields at least 85% of the initial protein. The residual $\leqq 15\%$ may consist either of irreversibly denatured material or of incompletely cross-linked protein shells (cf. optimization of cross-linking). The reassembly is found to be concentration dependent. The initial step is a fast dimer and trimer formation. At low protein concentrations, the dimer accumulates forming the most prominent intermediate species (Fig. 3). Monomers, dimers and trimers disappear in a synchronous fashion generating the dodecamer, i.e. the half-molecule of the complex as the final intermediate state. This accumulates to produce the native icositetramer.

After long incubation (> 30 min), a slight accumulation of higher polymers of apoferritin occurs; the amount never exceeds 20% of the total protein. In analyzing the kinetics, this portion was included in the native endproduct, because dimerization and even trimerization are known to be an inherent property of the native apoferritin complex. Polymerization may be favoured under the conditions of reconstitution and/or cross-linking (cf. Harrison and Gregory 1968; Stefanini et al. 1982).

The observed complex reconstitution pattern may be explained either by incomplete cross-linking of the intermediates on the assembly path, or by an equilibrium of monomers, dimers, trimers etc. in the process of structure formation. The first alternative is unlikely, because optimization experiments illustrated in Fig. 1 demonstrate that the monomer:dimer:trimer:icositetramer ratio is not altered at

varying glutaraldehyde concentrations. The second alternative suggests that the kinetic mechanism consists of a fast equilibrium of monomers, dimers and trimers, followed by the assembly to dodecamers (via hexamers) which finally dimerize in a rate-determining step.

Spectral analysis of the kinetics of renaturation and reassociation

The formation of "structured monomers" with proper reconstitution sites allowing unperturbed subunit assembly has been analyzed by pH and time dependent measurements of the far UV circular dichroism. The amplitude of the minimum at 222 nm reflects structural transitions indicating the loss of $\approx 15\%$ of the native helicity upon acid dissociation at pH 2.0 (Leach et al. 1976), and complete denaturation in 7.2 *M* G-HCl. The regain of the native state involves fast steps: at 22.6 µg/ml protein concentration, the recovery of $\approx 80\%$ of the final amplitude takes only 15 s. A subsequent slow phase points to rearrangements of the polypeptide backbone or additional secondary structure formation in the process of association. The kinetics of this slow phase coincide with the decrease in monomers, as monitored by cross-linking (Fig. 4).

Making use of the intrinsic tryptophan fluorescence, the regain of the native configuration may be monitored by an independent method, in addition to the CD kinetics. The intrinsic fluorescence of native horse spleen apoferritin is characterized by an emission maximum at 308–315 nm which is unusual for a tryptophan containing protein (Longworth 1971). Complete denaturation in G-HCl leads to the typical fluorescence emission of solubilized tryptophan residues at $\lambda_{max} = 350$ nm. Incubation of native apoferritin at pH 1.6–2.8 causes dissociation of the protein complex. At equilibrium, oligomers and subunits are present in concentrations depending solely on the pH of the medium.

In a similar way, the position of the fluorescence maximum of acid dissociated apoferritin is determined by the pH (Stefanini et al. 1982). Hence, λ_{max} may be used to characterize the state of association of the protein: with increasing disassembly, λ_{max} approaches its final value at 330 nm.

In the reassociation process, spectral shifts and changes in intensity are superimposed. The fast kinetic phase (which reaches its final value within 20 s) is accompanied by a significant blue shift of λ_{max} from 350 to 330 nm. This emission spectrum shows the characteristics of acid dissociated apoferritin (Crichton and Bryce 1973; Stefanini et al. 1982). This suggests that a mixture of "structured monomers" and small assembly products constitute an early intermediate state. Subsequent assembly to the native icositetramer is paralleled by a slow shift of λ_{max} to 315 nm, the limiting value characteristic for the native assembly. This process is monitored using the decrease of the fluorescence emission at 350 nm, and the kinetics reflect the rate-determining last association step on the assembly pathway, i.e. the formation of the "native" apoferritin complex. They parallel the formation of the icositetramer determined by the cross-linking technique (Fig. 4).

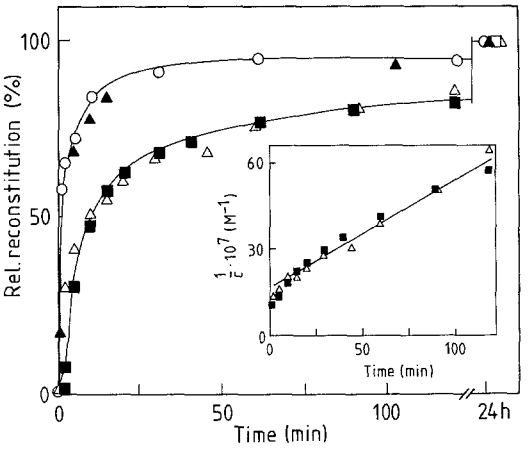

Fig. 4. Comparison of the reconstitution of apoferritin (22.6 µg/ml) as determined by fluorescence emission, dichroic absorption and cross-linking. The slow phase of the reassociation kinetics was calculated in relation to the final value for reconstituted (native) apoferritin. The kinetics were monitored making use of the decrease in fluorescence emission at 350 nm (△), as well as the slow phase of the increase in dichroic absorption at 222 nm (▲) (after the rapid regain of 80% of the final value). For comparison, data include the decrease in monomers (○) and the formation of the native apoferritin (■), determined by cross-linking. Each point represents the mean value of 3 independent determinations. *Insert:* Linearization of the kinetic profiles according to second order kinetics. Icositetramer formation from both cross-linking and fluorescence emission are characterized by one single rate constant $k_2 = 6.7 \cdot 10^4 M^{-1} s^{-1}$

Discussion

The acquisition of the native three-dimensional structure and the assembly to higher-order structures are primary functions of the nascent polypeptide chain. Their detailed mechanism is still not well-understood, mainly because available methods only allow the gross conformation to be analyzed. Determination of the association mechanism has become accessible by using chemical cross-linking in the process of reconstitution (Jaenicke and Rudolph 1986).

It has previously been shown that the self-assembly in vitro of apoferritin subunits after dissociation under strongly denaturing conditions does not require iron: reconstitution in the presence of the iron core yields empty apoferritin shells (Harrison and Gregory 1968). Attempts to unravel the underlying mechanism were unsuccessful. Neither the association intermediates nor the time course of association could be unambiguously determined (Crichton 1975; Sefanini et al. 1976, 1979).

In the present study, reconstitution was monitored by spectroscopic techniques and glutaraldehyde fixation of intermediates on the assembly pathway. The final product ($\approx 85\%$ of the starting material) resembles the native complex as far as particle weight and iron uptake are concerned (Gerl and Jaenicke 1987).

The assembly mechanism involves dimers, trimers, hexamers (in negligible amounts), and dodecamers as kinetic intermediates. To initiate reconstitution, the unfolded monomers have first to acquire a native-like conformation providing complementary interfaces for correct subunit recognition. As observed for a variety of proteins in previous investigations, this requirement is fulfilled in a fast reaction preceding the assembly process ($\mathscr{M}_1 \rightarrow M_1$) (cf. Jaenicke 1984). The "structured monomers" dimerize at high rate; since the reaction is fast compared with the subsequent association steps, the dimeric intermediate is accumulated at least at low protein cocentrations (Fig. 3).

From the crystal structure, the stability of the symmetrical dimer has been predicted (Banyard et al. 1978). Interactions over the three-fold axis would give rise to hexamers. Based on the present evidence it is formed via the trimer which is expected to be as stable as the tetramer, considering the arrangement of the subunits in the native apoferritin complex (cf. Ford et al. 1984). As shown in Figs. 2 and 3, hexamers could not be substantiated in significant amounts using the cross-linking method. Their occurrence was previously suggested based on fluorescence studies involving the binding of hydrophobic fluorophores to the apoferritin subunit (Stefanini et al. 1979). The apparent discrepancy may be explained in four different ways: *(i)* the fluorescent dyes used to monitor hexamer formation are known to stabilize hydrophobic interactions and may thus shift the trimer-hexamer equilibrium to the hexamer; *(ii)* the trimer-hexamer equilibrium may favour the trimer so that hexamers (formed either by trimer assembly or dodecamer disassembly) never reach significant concentrations; *(iii)* fast formation of the stable dodecamer from the hexameric intermediate may not allow accumulation of the latter species; *(iv)* the specific distribution of reactive lysine residues on the surface of the trimer or hexamer may not allow complete cross-linking (cf. Jaenicke and Rudolph 1986). The presence of significant amounts of low molecular weight particles observed in the optimization experiments (Fig. 1) may be considered to support the latter explanation.

The dodecamer occurs in significant amounts so that the overall mechanism may be described by the following scheme:

$$24\,\mathscr{M}_1 \rightarrow 24\,M_1 \rightleftharpoons 8\,M_1 + \\ + 8\,M_2 \rightleftharpoons 8\,M_3 \rightleftharpoons 4\,M_6 \rightleftharpoons 2\,M_{12} \rightleftharpoons M_{24}\,.$$

Since "structured monomers", dimers and trimers exhibit parallel concentration dependent decay kinetics, equilibria between these species are assumed. As indicated by a comparison of the residual amounts of incompletely cross-linked assembly products observed for the native protein (Fig. 1), and the final yields of intermediates in the assembly kinetics (Fig. 3), significant amounts of unspecific abortive species can be excluded.

At low protein concentrations ($< 5\,\mu g/ml$), deviations from simple second-order kinetics become obvious so that the overall reconstitution reaction must contain folding *and* association as rate-determining steps. Whether isomerization reactions at the level of the icositetramer, or lower levels of association are involved cannot be decided.

The given cross-linking data are corroborated by the characteristic changes in fluorescence emission observed upon reconstitution. As shown by Crichton and Bryce (1973) and Stefanini et al. (1982), the maximum of fluorescence emission (λ_{max}) depends on the state of association in that λ_{max} is shifted from 330 to 315 nm proportional to the amount of icositetramer present. Thus, studying the blue-shift during reconstitution provides a selective tool to measure icositetramer formation. In applying this approach, the reconstitution has been found to start from a mixture of "structured monomers" and assembly intermediates. The corresponding fluorescence emission spectrum shows the typical features of common tryptophan containing proteins. In regaining the native assembly structure, tryptophan (and tyrosine) residues are buried in the interior of the subunits and their interfaces, giving rise to the characteristic spectral changes accompanying the folding and association of the protein. Monitoring the decrease in the yield of fluorescence emission at 350 nm, the assembly mechanism deduced from cross-linking experiments is confirmed. Linearization of the increase in icositetramers and the decrease in fluorescence emission according to second-order kinetics, yields identical rate constants (Fig. 4, insert). This approximation considers only one single

rate-determining association reaction, neglecting folding processes at the monomer level.

In order to separate the recovery of the backbone structure of the subunits from the assembly process, the regain of the native dichroic absorption was measured. Again, the kinetic profiles show two phases (Fig. 4). An initial fast one ($\approx 80\%$) reflects the regain of most of the final backbone structure. Similar fast folding reactions have been commonly observed as the first step in the "consensus pathway" for protein folding described by Goldberg (1985) (Jaenicke 1987). The regain of the residual $\approx 20\%$ coincides with the decrease in monomer concentration (as obtained from cross-linking), thus indicating that "shuffling" at the level of the assembly intermediates or the icositetramer is connected with measurable changes in secondary structure.

In contrast to complex assembly systems like phages or ribosomal particles, apoferritin does not require specific factors or assembly programs for its self-assembly. The only requirement for the autonomous and spontaneous formation of the native quaternary structure is the unperturbed amino acid sequence and its folding to "structured monomers". As shown by the present cross-linking experiments and spectral data, they provide the recognition sites that finally lead to the native protein shell. The in vitro assembly is found to be a sequential process involving dimers, trimers and dodecamers as intermediates. The in vivo assembly may be assumed to follow the same pathway.

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