Self-Assembly of Apoferritin from Horse Spleen after Reversible Chemical Modification with 2,3-Dimethylmaleic Anhydride†

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Appendix: Self-Assembly Intermediates of Horse Spleen Apoferritin Considered in Relation to a Structural Model

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ABSTRACT: Apoferritin from horse spleen is composed of 24 subunits that undergo partial dissociation after chemical modification with 2,3-dimethylmaleic anhydride (DMMA), yielding dimeric, trimeric, and tetrameric intermediates, stable at pH 8.5 and 0 °C. Deacylation at neutral pH and elevated temperature provides a means to initiate reassembly by appropriate shifts of the solvent conditions. In order to monitor the pathway of self-assembly, starting from different intermediates of dissociation, dimers, trimers, and tetramers were isolated and investigated with respect to their capacity to accomplish reassociation. Intrinsic protein fluorescence, gel permeation chromatography, and analytical ultracentrifugation were applied to characterize the intermediate and final stages of association. The assembly of both the dimer and trimer yields >85% of the native tetracosamer; the overall rate, starting from the dimer, exceeds the one starting from the trimer. Under comparable conditions, the tetramer exhibits only partial reassociation via the dimer and monomer; the corresponding dissociation reaction determines the observed slower rate. Significant assembly intermediates are "structured monomers", dimers, trimers, and dodecamers. Polymerization of the dimer via the tetramer, octamer, etc., does not occur on the pathway of assembly. The results confirm the assembly scheme proposed previously on the basis of cross-linking and spectroscopic experiments [Gerl, M., & Jaenicke, R. (1987) *Eur. Biophys. J. 15*, 103–109]. Comparison of structural models involving the different subunit interactions responsible for the sequential association supports the monomer → dimer → trimer → hexamer → dodecamer → tetracosamer mechanism of apoferritin self-assembly.

Apoferritin, the major iron storage protein in eukaryotic cells, is composed of 24 structurally equivalent subunits in *F*432 symmetry. In the cagelike shell of the protein (M_r 476 000) each subunit makes contact with five neighbors. The predominant structural units of the rhombic dodecahedron are dyad-related subunits that constitute one rhomb face. The apices of the rhombs touch at the threefold and fourfold axes of the molecule. Interactions around these axes result in two different channels that penetrate the protein shell (Rice et al., 1983; Ford et al., 1984).

From the crystal structure of the native protein, high stability of the symmetrical dimer has been predicted (Banyard et al., 1978). Further assembly of the complete protein shell has been assumed to proceed by the polymerization of the dimeric protomer via the tetramer and hexamer. This symmetrical entity which is stabilized by interactions around the threefold axis could then assemble to complete the shell [cf. Crichton (1975), Banyard et al. (1978), Clegg et al. (1980), and Ford et al. (1984)]. A similar polymerization mechanism involving dimers, tetramers, and octamers has recently been proposed by Stefanini et al. (1987). In contrast to both of the aforementioned hypotheses, previous cross-linking and spectroscopic experiments in our laboratory provided clear evidence for a sequential kinetic scheme involving "structured monomers", dimers, trimers (hexamers), and dodecamers as assembly intermediates (Gerl & Jaenicke, 1987b). The final product proved to be the tetracosamer in its native state (Gerl & Jaenicke, 1987a).

Apart from chemical cross-linking, fluorescence emission and circular dichroism were applied to characterize the assembly pattern. Through these approaches it has been possible to monitor the monomer → dimer → trimer transition as well as the rate-determining formation of the native tetracosamer (Gerl & Jaenicke, 1987b). However, incomplete cross-linking of the native protein complex and the rather low proportion of the trimeric intermediate in the process of reassociation lead to a certain ambiguity with respect to the trimeric species which—instead of an assembly intermediate—might represent an artifactual dissociation product of incompletely cross-linked intermediates, including the tetracosamer. For this reason, independent methods to confirm the above assembly mechanism are desirable.

In the present study, chemical modification with 2,3-dimethylmaleic anhydride was used to analyze separate association reactions of the dimeric apoferritin protomer, as well as the trimer and tetramer. This reagent is known to partially disassemble protein complexes. Dixon and Perham (1968)

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were able to show that the derivatives obtained at pH >8.5 (0 °C) are unstable at neutral pH and elevated temperature. Thus intermediates of disassembly may be easily separated and subsequently reconstituted by shifting the pH and temperature. Owing to the mild acylation–deacylation conditions, apoferritin maintains its native secondary structure in the disassembly and reassembly processes so that the mechanism of reassociation is expected to be unperturbed by significant folding steps.

Materials and Methods

Substances. Ferritin from horse spleen was purchased from Boehringer, Mannheim, dithioerythritol (DTE)[1] was from Roth, Karlsruhe, guanidinium chloride (GdmCl), ultrapure, was from Schwarz-Mann, Orangeburg, NY, 2,4,6-trinitrobenzenesulfonate (TNBS) was from Fluka, Basel, maleic anhydride, 2,3-dimethylmaleic anhydride (DMMA), and ϵ-amino-*n*-caproic acid were from Sigma, München, Sephadex G-75 from Pharmacia, Uppsala, and AcA 54 Ultrogel was from LKB, München. All other reagents were A-grade substances from Merck, Darmstadt. Quartz-bidistilled water was used throughout. Standard buffer was 0.1 M sodium phosphate, pH 8.5. For preparation of apoferritin, see Gerl and Jaenicke (1987a).

Maleylation of lysine residues followed the procedure of Butler et al. (1969). Maleic anhydride was used as 0.8 M solution in absolute ethanol. Apoferritin at concentrations 5–10 mg/mL in 0.3 M sodium phosphate, pH 8.5, 0 °C, was treated with a 60-fold molar excess of reagent. After being stirred for 30 min at 0 °C, the reaction was terminated by dialysis against 0.1 M sodium phosphate, pH 8.5, 1 mM EDTA, and 1 mM DTE.

Estimation of the degree of modification by reaction with TNBS followed the method of Habeeb (1966) by determining the absorbance of the lysine derivatives at 335 nm (Shimadzu-UV 110-02 spectrophotometer), and by comparison with a calibration curve with ϵ-amino-*n*-caproic acid.

The stability of native apoferritin (at pH 7.3 and 4.2, respectively) and the maleylated protein (at pH 7.3) was measured by making use of the fluorescence-detected equilibrium transition at varying GdmCl concentrations (c_p = 25 μg/mL, 20 °C). Determination of the intrinsic fluorescence emission at 280–360 nm (λ_{ex} = 280 nm) was performed after 24-h incubation (Hitachi/Perkin-Elmer MPF 44A spectrofluorometer, equipped with a corrected spectra accessory). To monitor the degree of denaturation, the ratio of fluorescence emission at 315 and 350 nm ($F_{315/350}$) was applied (Gerl & Jaenicke, 1987a).

Modification of apoferritin with DMMA was accomplished by treating the native protein (c_p = 5–30 mg/mL in 0.3 M sodium phosphate, 3 mM EDTA) with a 60-fold molar excess of DMMA. The modifying reagent was added in small portions, either in solid form or solubilized in dioxane (300 mg/mL in 50-μL portions) at 0 °C. The pH of the reaction mixture was kept constant at pH 8.6 ± 0.4 with 2 N NaOH. Dissociation of the apoferritin complex was estimated from the change in intrinsic fluorescence ($F_{315/350}$). After the $F_{315/350}$ had reached ≈25% of the value observed for native apoferritin, the modification reaction was stopped by dialysis against 0.1 M sodium phosphate, pH 8.5, 1 mM EDTA, and 1 mM DTE at 4 °C.

[1] Abbreviations: c_p, protein concentration; DMMA, 2,3-dimethylmaleic anhydride; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; HPLC, high-performance liquid chromatography; RNase, bovine pancreatic ribonuclease; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

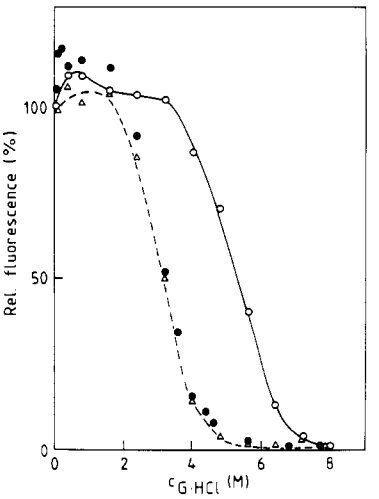

FIGURE 1: Guanidine denaturation of native and maleylated apoferritin in 0.1 M sodium phosphate buffer, containing 1 mM EDTA and 1 mM DTE, 20 °C. Fluorescence ratio $F_{315/350}$ at λ_{ex} = 280 nm, measured after 24-h incubation at 20 °C. Apoferritin at pH 7.3 (○) and 4.2 (△). Maleylated apoferritin (modified at four to five lysine residues per subunit) at pH 7.3 (●).

Separation of the dissociation intermediates made use of gel permeation chromatography (Sephadex G-75, 2.2 × 55 cm column, or AcA 54 Ultrogel, 2.2 × 60 cm and 2.8 × 85 cm columns) at pH 8.5, 4 °C.

Cleavage of DMMA and subsequent reassocation of apoferritin was achieved by incubating aliquots of the isolated dissociation intermediates in 0.1 M sodium phosphate buffers containing 1 mM EDTA and 1 mM DTE at pH 2.0, 6.6, 7.5 and 8.5, respectively (20 °C). The degree of reassociation was analyzed by fluorescence spectroscopy, gel filtration (AcA 54 Ultrogel, 2.2 × 60 cm column), or HPLC (LKB-G 3000 SW, 0.75 × 60 cm column, and TSK G-SW, 0.75 × 7.5 cm precolumn), as well as analytical ultracentrifugation.

Ultracentrifugation made use of a Beckman Spinco E analytical ultracentrifuge equipped with a high-intensity light source and a UV scanning system. Double-sector cells with sapphire windows were used in an An-G rotor. Measurements were performed at 20 °C. For sedimentation equilibrium the meniscus depletion technique was applied (Yphantis, 1964).

HPLC measurements were performed by using an LKB-2156 solvent conditioner, LKB-2152 controller, and LKB-2150 pump. To monitor the eluent, a Merck/Hitachi F-1000 fluorescence detector (λ_{ex} = 280 nm, λ_{em} = 320 nm) was applied.

Results

Modification of Apoferritin with Maleic Anhydride. Comparison of the stabilities of native apoferritin and the protein maleylated at four to five lysine residues per subunit shows that the midpoint of the guanidine-dependent denaturation equilibrium transition at pH 7.3 is shifted from 5.4 to 3.2 M GdmCl (Figure 1). The profile of the maleylated protein coincides with that of native apoferritin at pH 4.2, i.e., close to its isoelectric point. Scatter of the data at low GdmCl concentrations reflects the influence of maleylation on the fluorescence properties of the native protein; in the transition range, the error is small so that, as in the case of the native protein, the dissociation of the modified protein may be characterized by the change in $F_{315/350}$ [cf. Gerl and Jaenicke (1987a)].

Modification of Apoferritin with DMMA: Dissociation of Apoferritin by Coupling with DMMA. As shown in Figure

Table I: Reassociation of Intermediates on the Assembly Pathway of Apoferritin after DMMA Coupling and Subsequent Deacylation at Varying pH and Temperature[a]

	dimer (20 °C)						tetramer (20 °C)						trimer (0 °C)			
	pH 2.0		pH 6.6		pH 8.6		pH 2.0		pH 6.6		pH 8.6		pH 7.5		pH 8.6	
t	F	λ	F	λ	F	λ	F	λ	F	λ	F	λ	F	λ	F	λ
0	20	328	20	328	20	328	10	330	10	330	10	330		329		329
0.02	23	327	18	328	17	328	22	327	12	330	10	332				
1													31	327		
2	22	327	69	312	18	328	19	328	19	330	10	332	40	320		
3													55	315		
4													60	315		
20	20	327	82	310	26	327	19	328	32	325	15	330	60	314	18	329
48	24	326	98	308	58	315	26	327	35	325	26	328			20	328
96					85	310			36	324			58	314		

[a] Intrinsic fluorescence was used to characterize the state of association at varying times (t). t, time in hours; λ, maximum of fluorescence in the corrected emission spectrum (λ_{ex} = 280 nm); F, ratio of fluorescence emission ($F_{315/350}$) in percent of the value observed for native apoferritin. The association of apoferritin is accompanied by a blue shift of the fluorescence maximum from ≈330 to ≈310 nm and by a simultaneous increase of the fluorescence ratio $F_{315/350}$: (i) λ_{max} = 329 ± 2 nm is a value characteristic for low molecular weight intermediates of apoferritin obtained after acid dissociation or after short-term reassembly (Gerl & Jaenicke, 1987a,b); (ii) λ_{max} = 310 ± 2 nm is the fluorescence maximum of the native apoferritin complex [cf. Crichton and Bryce (1973), Stefanini et al. (1982), and Gerl and Jaenicke (1987a,b)].

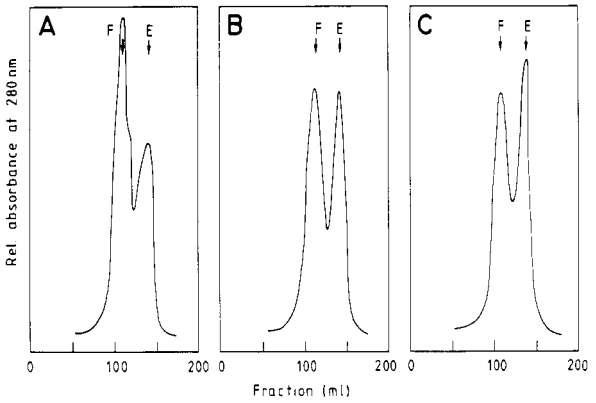

FIGURE 2: Disassembly of apoferritin by maleylation with DMMA, as monitored by gel permeation chromatography on Sephadex G-75 (2.2 × 55 cm). After $F_{315/350}$ had reached 47% (A), 35% (B), and 31% (C) of the value observed for native apoferritin, derivatization was stopped by dialysis against 0.1 M sodium phosphate buffer, pH 8.5. Marker proteins: F, ferritin (M_r 450K), E, egg albumin (M_r 45K).

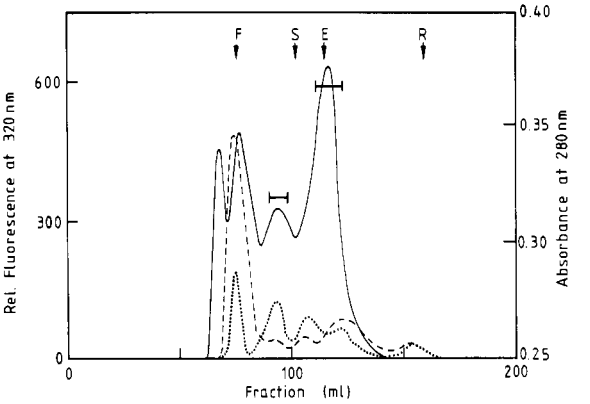

FIGURE 3: Separation of the chemically modified disassembly and reassembly products of apoferritin by gel permeation chromatography on AcA 54 Ultrogel (2.2 × 60 cm), in 0.1 M sodium phosphate buffer, pH 8.5. For disassembly (—), apoferritin was reacted with a 60-fold molar excess of DMMA (see Materials and Methods). Left ordinate: Fluorescence emission at 320 nm (λ_{ex} = 280 nm). For reassembly, pooled and concentrated dimer and tetramer fractions (indicated by ⊢⊣) were incubated for 80 h at pH 8.5 (20 °C) and subsequently subjected to gel filtration: dimers (---); tetramers (···). Right ordinate: Absorbance at 280 nm. Marker proteins: F, ferritin (M_r 450K); S, bovine serum albumin (M_r 66K); E, egg albumin (M_r 45K); R, bovine pancreatic RNase (M_r 14K).

2, apoferritin may be dissociated by coupling with 2,3-dimethylmaleic anhydride. The degree of dissociation during the reaction was estimated from the change in intrinsic protein fluorescence. The derivatization reaction was terminated after the fluorescence ratio $F_{315/350}$ of the protein had reached ≈47%, 35%, and 31% of the value observed for native apoferritin (panels A, B, and C of Figure 2). The dissociation products are found to be stable at pH 8.5, 0 °C, for >72 h (cf. Figures 4 and 5); hence, they may be separated by gel permeation chromatography. As is obvious from the profiles, the dissociation of the apoferritin complex is accompanied by a decrease in the fluorescence ratio $F_{315/350}$.

In order to achieve dissociation intermediates on a preparative scale, DMMA derivatization was performed in 0.8 M DMMA in dioxane at pH 8.5–9.0. The amount of dioxane never exceeded 10%, in order to preclude dissociation of the native apoferritin complex. The reaction was stopped after the $F_{315/350}$ ratio had reached ≈25% of the value observed for the native apoferritin complex. The separation of the chemically modified dissociation products by gel filtration on AcA 54 Utrogel is illustrated in Figure 3. The profile clearly indicates that the reaction yields dimers and tetramers, apart from intact tetracosamers and higher aggregates. When the fractions indicated by the bars are used, the dimer is homogeneous: Its molecular weight and its stability are confirmed by HPLC and sedimentation analysis (see below). The tetramer fraction may be contaminated by trimers. The sedimentation boundary ($s_{20,w}$ = 5.0 ± 0.4 S) does not show significant trailing toward the meniscus so that the amount of trimers cannot exceed 10%. As proven by the spectral properties summarized in Table I, both fractions show high long-term stability at pH 8.6, 20 °C, for at least 20 h.

In order to avoid effects of the nonpolar solvent component, an alternative method of preparative dissociation made use of the stepwise addition of solid DMMA in small portions at pH 8.2–8.5, 0 °C. After 60-fold molar excess of reagent was added, the derivatization was stopped by dialysis against 0.1 M sodium phosphate, pH 8.5, containing 1 mM EDTA and 1 mM DTE (0 °C). Half of the concentrated protein solution was passed through an AcA 54 Ultrogel column (2.8 × 85 cm) after 2-h dialysis; the other half, after 20 h. Both samples yielded dimeric and trimeric dissociation products (data not shown).

Reassociation of Dimers and Tetramers after DMMA Coupling in the Presence of Dioxane. The N^{ϵ}-acyl bond between lysine residues and DMMA is prone to hydrolysis so that cleavage of the lysine–DMMA bond may be accomplished under mild solvent conditions. Since the rate of deacylation

depends strongly on pH and temperature, reassociation of the chemically modified protein may be accomplished by shifting the pH and temperature of the medium. Under optimum conditions, the self-assembly of the protein is limited by the rate of the uncoupling reaction which shows its maximum at neutral pH. In order to analyze the reassociation of assembly intermediates of apoferritin, separate peak fractions (indicated in Figure 3) were incubated in sodium phosphate buffer in the presence of 1 mM EDTA and 1 mM DTE at pH 2.0, 6.6, and 8.5 (20 °C). The results are illustrated in Table I and Figure 3. Complete regain of the fluorescence ratio of native apoferritin is only observed for the dimer fraction. Fractions containing mainly the tetramer (>75%) show partial disassembly to dimers and monomers, apart from a relatively low final yield of tetracosamers. Obviously, the fluorescence and assembly characteristics of the tetramer fraction differ significantly from those of the dimer. The fluorescence emission shows a significant red shift, which indicates weaker subunit interactions compared to both the dimer and tetracosamer. Under no conditions of reassembly, the tetramer reaches the fluorescence ratio observed for native apoferritin, in accordance with the fact that the assembly pathway starting from the tetramer must be blocked. The rate of the fluorescence change differs widely at the given pH values. At pH 2.0, neither denaturation nor association can be detected; the fluorescence ratio remains unchanged within 48 h, showing the spectral characteristics typical for low molecular weight intermediates (preferentially dimers) [cf Crichton and Bryce (1973) and Stefanini et al. (1982)]. At pH 6.6, release of DMMA and subsequent reassociation are fast while at pH 8.5, a lag phase in the fluorescence kinetics points to an increase in stability of the lysine–DMMA bond. In order to characterize the final particle distribution (after reassembly has approached equilibrium), pooled and concentrated dimer and tetramer fractions were incubated for 80 h at pH 8.5 (20 °C).

Optimum homogeneity of the starting material was accomplished by taking peak fractions only (cf. Figure 3). Ultracentrifugal analysis of these fractions yielded $s_{20,w} = 3.5 \pm 0.3$ S for the dimer and $s_{20,w} \approx 5$ S for the tetramer fraction.

At the given pH and temperature, the hydrolysis of the Lys–DMMA bond is exceedingly slow. Since the process of reassociation is triggered by this cleavage reaction, the particle distribution can be analyzed by gel permeation chromatography on an AcA 54 Ultrogel column (Figure 3).

Starting from the dimer, the ratio of monomeric intermediates, unreacted dimers, trimers, tetramers, and tetracosamers after 80-h incubation is of the order of 5:20:10:65. After 100 h, reconstitution reaches completion with ≈85% native tetracosamer and <15% higher polymers. These results are in agreement with the conclusions drawn from fluorescence emission as well as chemical cross-linking [cf. Gerl and Jaenicke (1987b)].

In contrast to the high efficiency of the dimer → tetracosamer assembly, the tetramer fraction yields equal amounts of dimers, trimers, tetramers, and tetracosamers, apart from ≈5% monomers. The result indicates clearly that in this case reassociation is impeded due to a "disproportion mechanism" involving dissociation to dimers and monomers and subsequent slow (incomplete) reassembly (Figure 3). Even under conditions favoring the cleavage of the lysine–DMMA bond (pH 6.6) and enhancing reassociation, the tetramer fraction is uncapable of recovering the native tetracosamer at high yield (cf. Table I). In summarizing the results, the reconstitution profiles prove the self-assembly of apoferritin to involve monomers, dimers, and trimers. The tetrameric intermediate of

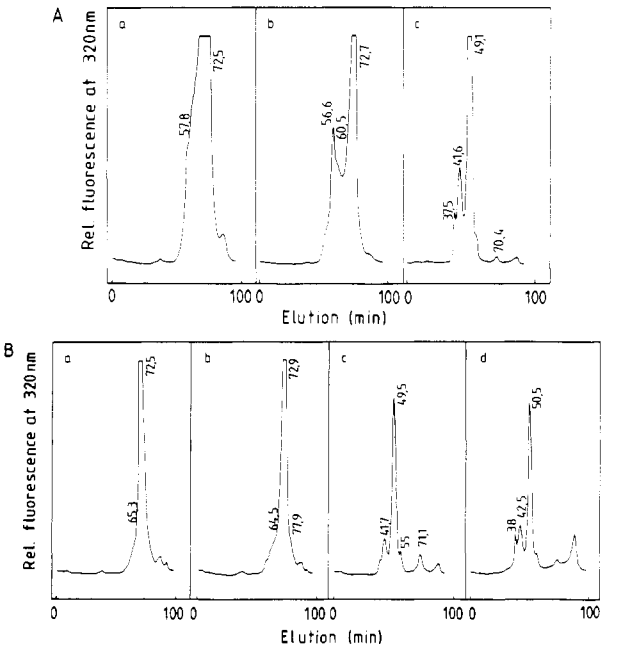

FIGURE 4: Reassembly of the dimeric dissociation product of DMMA-modified apoferritin, as monitored by HPLC (see Materials and Methods). Elution buffer: 0.1 M sodium phosphate, 0.1 M Na_2SO_4, pH 6.5; p = 12 bar, flow rate = 0.3 mL/min. Analysis of the particle distribution in fractions of the DMMA-modified protein separated after 2-h (A) and 20-h (B) dialysis against standard buffer (0.1 M sodium phosphate, pH 8.5). Stability of the dimeric intermediate was confirmed by 48-h incubation in standard buffer at 0 °C (a). pH shifts to pH 7.5 and 6.5 and 1-h incubation at the given pH values (0 °C) lead to deacylation accompanied by reassociation to particle distributions given in (b) and (c), respectively. Long-term incubation (20 h) at pH 6.5 (0 °C) yields the pattern of native apoferritin (d). Calibration of the column with ferritin, mitochondrial malate dehydrogenase, octopine dehydrogenase, soybean trypsin inhibitor, RNase, and lysozyme yields the following retention times for apoferritin intermediates: M_1, 76–79 min; M_2, 70–72 min; M_3, 66–68 min; M_4, 62–65 min; M_6, 59–61 min; M_{12}, 51–55 min; and M_{24}, 48–51 min.

disassembly does not seem to be a genuine assembly intermediate since (i) the dimeric sample does not yield tetramers and (ii) the assembly starting from the tetramer is exceedingly slow and occurs only in low yields. Tetracosamers observed in this fraction may be the result of either a disproportionation reaction of tetramers or the assembly of trimers contaminating the tetramer fraction.

Reassociation of Dimers and Trimers after Coupling with Solid DMMA. Results of assembly experiments starting from the trimer are illustrated in Table I. As in the case of the tetrameric intermediate, the change in $F_{315/350}$ after lowering the pH is slow and does not lead to complete reassembly within reasonable time. The recovery amounts to ≈60% after 90-h incubation, compared with ≈100% in the dimer fraction and ≈35% in the tetramer fraction. The characteristics of the dimer and its product of reassembly are the same as those observed for the protein modified in the presence of dioxane. At pH 8.5 and 0 °C, the modified protein is stable over long periods of time; only upon cleavage of the N^{ϵ}-acyl bond, the fluorescence characteristics change at a rate paralleled by alterations in the particle distribution (cf. Table I). Corresponding HPLC analyses depicting the particle distribution after 2- and 20-h dialysis and subsequent separation by gel permeation chromatography are shown in Figures 4 and 5 to illustrate the reassociation of the DMMA-modified dimeric and trimeric dissociation products. As mentioned previously, the time of dialysis influences the particle distribution in the dimer fraction. In the sample separated after 2-h dialysis,

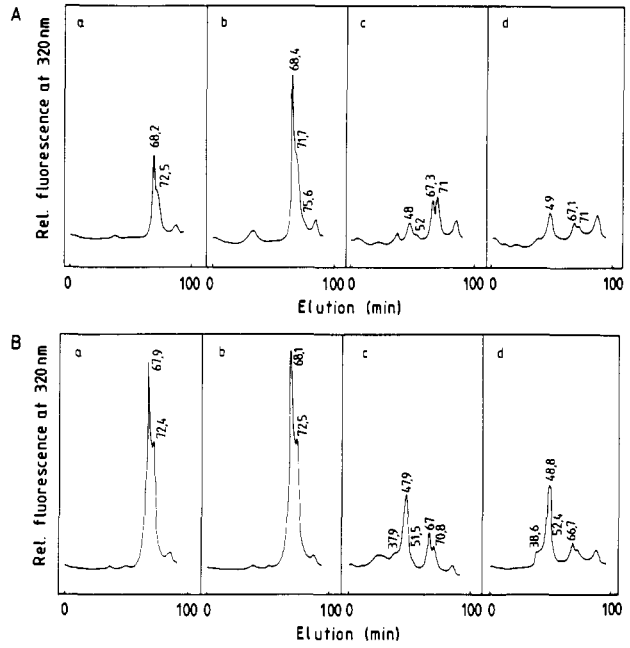

FIGURE 5: Reassembly of the trimeric dissociation product of DMMA-modified apoferritin as monitored by HPLC. For further details, see Figure 4.

significant amounts of intermediates other than the dimer occur, whereas after 20-h dialysis >90% dimers are obtained. This observation suggests that, after short dialysis, there are numerous incompletely modified dimers which, after separation, slowly regain their capacity to reassociate to intermediates beyond the dimer. After long incubation, these intermediates can be separated, yielding the dimer as a homogeneous fraction.

The long-term stability of the N^{ϵ}-acyl bond at pH 8.5, 0 °C, is confirmed by HPLC (frames a in Figures 4 and 5). Lowering of the pH to pH 6.6 initiates rapid association. Because of the high rate of the assembly reaction, it is not possible to quantify the intermediates of association in an unambiguous way. Split bands in Figure 4 (frame b) point to monomers, dimers, trimers, hexamers, and dodecamers as assembly intermediates.

The trimer is less stable than the dimer. Its dissociation at pH 8.5, 0 °C, leads to an equilibrium with a significant dimer population (Figure 5). Upon a shift in the pH to pH 6.6, slow reassociation to form the native tetracosamer is observed. As in the case of the tetramer, the reassembly is incomplete and slow compared to the rate observed in the dimer → tetracosamer assembly. During the whole association reaction the trimer concentration equals or even exceeds the concentration of the dimer, in contrast to previous cross-linking results (Gerl & Jaenicke, 1987b). These differences may be explained by the assumption that the observed trimers do not represent the genuine assembly intermediate. DMMA-induced disassembly may favor intermediates stabilized by polar interactions not involving ϵ-amino groups. From the crystal structure (Banyard et al., 1978; Rice et al., 1983), a plausible candidate could be the propeller-like trimer around the threefold axis of the protein. Due to the contribution of the stable antiparallel related dimeric intermediate, this species is unable to reassemble to the native protein.

In order to substantiate the chromatographic determinations of the assembly pattern, dimeric and trimeric dissociation intermediates were analyzed in the analytical ultracentrifuge. Samples were incubated in sodium phosphate, pH 8.2 at 20 °C; under these conditions, association is slow enough to be accessible to sedimentation analysis. At sedimentation equilibrium, an approximate determination of assembly intermediates is possible.

Table II: Reassembly of the Dimeric and Trimeric Dissociation Products of DMMA-Modified Apoferritin As Monitored by Ultracentrifugation[a]

	dimer		trimer	
	A	B	A	B
$s_{20,w}$ (S)[b]	1.6		2.0	2.2
	11.0	13.1	3.6	3.2
			13.0	12.2
M_w ($\times 10^{-3}$)[c]	33 ± 1	34 ± 1	40 ± 10[d]	40 ± 10[d]
	430 ± 12	510 ± 15	180 ± 10	271 ± 10

[a] Analysis of the intermediary particle distributions in fractions of the DMMA-modified protein separated after 2-h (A) and 20-h (B) dialysis against standard buffer (cf. HPLC analysis in Figures 4 and 5). [b] Evaluation of the separate steps of the sedimentation profiles by plotting log r_i vs t allows one to discriminate monomers (≈2 S), dimers (≈3.6 S), and dodecamers (≈12 S). [c] M_w values represent weight averages of the particle distribution during the reassembly process paralleling the cleavage of the DMMA–protein bond. By use of the short column overspeed technique, and going from higher to lower rpm values, quasi-equilibria were accomplished after ≤12 h. [d] Average from nonlinear ln y vs r^2 plots; high-speed sedimentation equilibria applying meniscus depletion conditions yield the minimum molecular weight.

Sedimentation velocity runs at high speed exhibit multistep profiles indicating defined and separable particles. The corresponding sedimentation coefficients are summarized in Table II. $s_{20,w} \approx 2$ S may be assumed to characterize the apoferritin subunit. The presence of a 2S species supports a mechanism involving the monomer. The fraction characterized by $s_{20,w} \approx 3.6$ S may be ascribed to the dimeric intermediate (Crichton & Bryce, 1973; Stefanini et al., 1979, 1982, 1987), while $s_{20,w} \approx 12$ S corresponds to an assembly intermediate of the size of the dodecamer.

High-speed sedimentation equilibria based on the meniscus depletion technique (Yphantis, 1964) basically allow a detailed population analysis during the slow assembly process. With the present broad particle distribution only components with particle weights below the dodecamer can be evaluated. The results confirm an association scheme involving the dimer, trimer, and dodecamer as intermediates (Table II). Tetramers and octamers were not detectable. The limiting value of ≈270K in the bottom region of the cell cannot be ascribed to a well-defined assembly intermediate. It rather represents the weight average of the various disassembly products and native apoferritin; higher aggregates may contribute after extended equilibration.

After equilibrium centrifugation, the protein in its final state of association was analyzed by HPLC. The recovery of the native protein complex exceeded 85%; higher aggregates were below 15%.

Discussion and Conclusions

Apoferritin from horse spleen shows unusual stability and may be dissociated only under very strong denaturing conditions (Harrison & Gregory, 1968; Smith-Johannsen & Drysdale, 1969; Listowsky et al., 1972; Crichton & Bryce, 1973). According to previous reports, incubation of the native protein at pH <3.0 leads to an equilibrium between the native tetracosamer and its subunits (Crichton, 1972; Crichton & Bryce, 1973; Stefanini et al., 1982). The degree of dissociation depends on the pH and can be estimated either from the red shift of the fluorescence maximum (Stefanini et al., 1982) or from the ratio of fluorescence emission $F_{315/350}$ (Gerl & Jaenicke, 1987a). Since the pH-dependent equilibrium yields all intermediates of dissociation, no stable and homogeneous

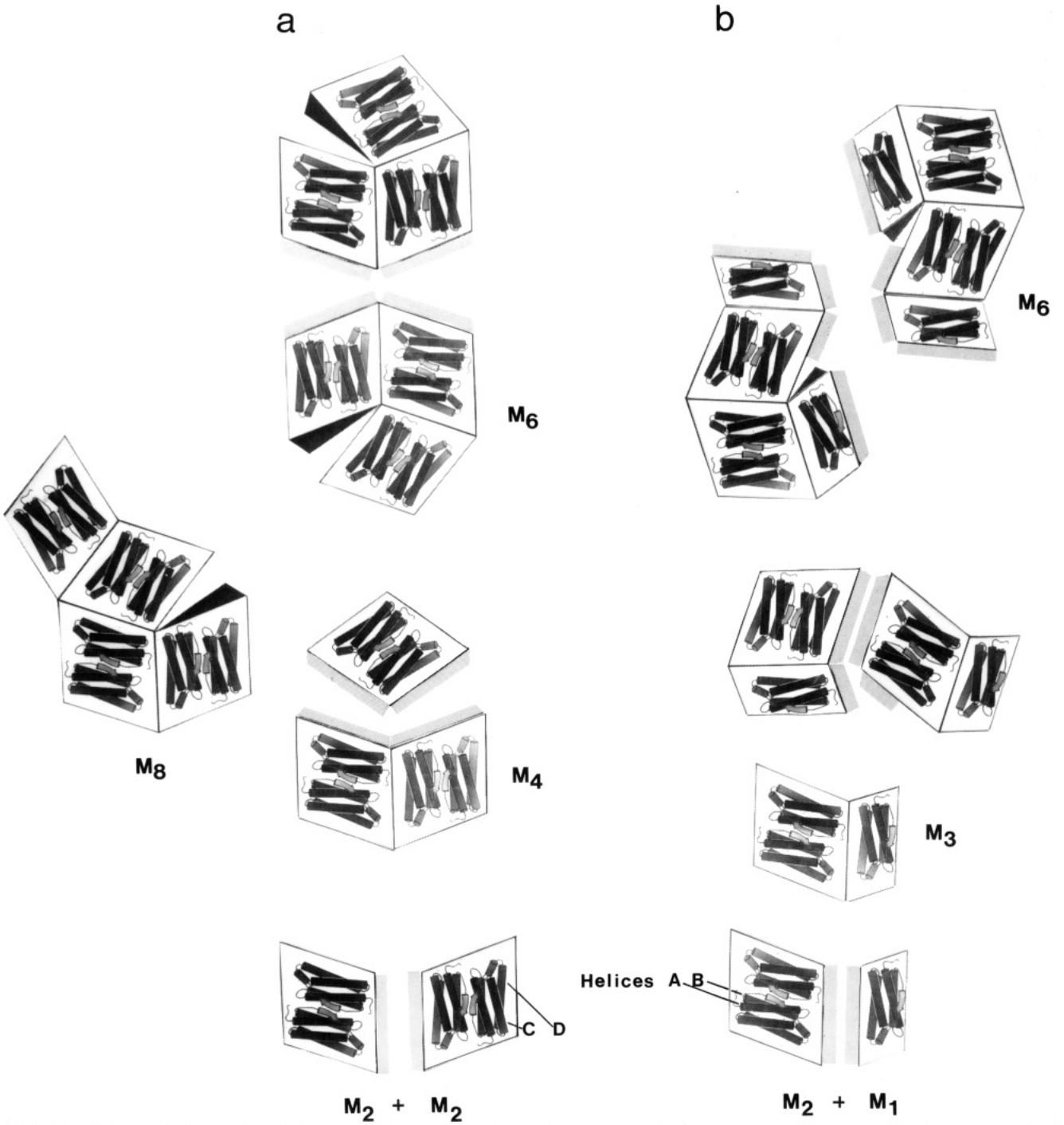

FIGURE 6: Schematic representation of the assembly mechanism of apoferritin. (a) Intermediates of the hypothetical mechanism including dimeric and tetrameric intermediates and the hexamer as a trimer of dimers. (b) Intermediates of the present mechanism including dimeric and trimeric intermediates and the hexamer as a dimer of trimers. The hatched welts symbolize the possible interactions to generate the next assembly intermediate along the sequence; the black welts in the cutout model represent the interactions already realized within the intermediate. M_i, intermediates of association with i = number of subunits.

dissociation intermediates can be isolated upon acid dissociation.

In contrast to the previously described monomer–multimer equilibrium, chemical modification of protein amino groups with DMMA is known to yield stable disassembly intermediates (Dixon & Perham, 1968; Gibbons & Perham, 1970; Means & Feeny, 1971; Puigserver & Desnuelle, 1975). The dissociation of the subunits is caused by electrostatic interactions apart from the hydrophobic effect of the methyl groups of DMMA. The fact that Coulomb interactions alone are not sufficient for the disassembly of apoferritin is clearly established by the effect of maleic anhydride: the derivatized protein still shows the capacity to oxidize Fe^{2+}, thus proving its intact quaternary structure (Wetz & Crichton, 1976).

The covalent modification of lysine residues with 2,3-dimethylmaleic anhydride has been previously applied to dissociate oligomeric or multimeric protein complexes (Dixon & Perham, 1968). The present study of apoferritin from horse spleen shows that, depending on the solvent conditions, dimers, trimers, and tetramers can be isolated as intermediates of disassembly. At low temperature and slightly alkaline pH they show long-term stability. Shifting the solvent conditions to neutral pH and/or 20 °C favors deacylation so that reassembly of the native protein complex takes place. Due to the mild

conditions of the reaction, the protein reassociates more or less in its unperturbed backbone structure. Therefore, starting the association reaction from the above-mentioned intermediates, the assembly pathway can be analyzed without significant competition by folding or "wrong aggregation" (Jaenicke, 1984, 1987).

As a consequence, the yield of reconstituted apoferritin exceeds 80%; the amount of polymers (which are also present in native material) is of the order of 15%.

Numerous attempts have been made in the past to elucidate the assembly mechanism of the apoferritin complex. No conclusive model could be derived from spectroscopic, hydrodynamic, and chromatographic measurements. Previous cross-linking experiments and spectral studies pointed to a sequential reaction mechanism with the dimer, trimer, and dodecamer as well-populated intermediates (Gerl & Jaenicke, 1987b).

The present reassociation experiments confirm the assembly pathway in a direct approach by starting the reaction from isolated "structured intermediates". In summarizing the results, we may conclude that the native tetracosamer is formed in a sequence of well-defined steps involving the dimer, trimer, hexamer, and dodecamer:

$$24\mathcal{M}_1 \rightarrow 24M_1 \rightleftharpoons 8M_2 + 8M_1 \rightleftharpoons 8M_3 \rightleftharpoons (4M_6) \rightleftharpoons 2M_{12} \rightleftharpoons M_{24}$$

where $\mathcal{M}_1$ = unfolded subunit, M_i = assembly intermediates with i subunits, and M_{24} = native tetracosamer. The latter has been previously demonstrated to be indistinguishable from the native starting material in its structural and functional properties (Gerl & Jaenicke, 1987a).

As in the case of the cross-linking data, the present results give clear evidence for an equilibrium between structured monomers, dimers, and trimers preceding dodecamer and tetracosamer formation. Hexamers are not detectable in significant amounts, again in agreement with the cross-linking pattern; obviously, their dimerization to the dodecamer is fast. Tetrameric and octameric assembly intermediates were also not observed, neither by gel permeation chromatography nor by ultracentrifugation; this is in contrast to conclusions reported by Stefanini et al. (1987).

As shown in the Appendix, the given assembly mechanism is in agreement with predictions based on the crystal structure of the protein (Banyard et al., 1978; Clegg et al., 1980).

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References

Banyard, S. H., Stammers, D. K., & Harrison, P. M. (1978) *Nature (London) 271*, 282–284.

Butler, P. J. G.; Harris, J. J., Hartley, B. S., & Leberman, R. (1969) *Biochem. J. 12*, 679–689.

Clegg, G. A., Fitton, J. E., Harrison, P. M., & Treffry, A. (1980) *Prog. Biophys. Mol. Biol. 36*, 53–86.

Crichton, R. R. (1972) *Biochem. J. 130*, 35P.

Crichton, R. R. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., Ed.) pp 253–260, North-Holland, Amsterdam.

Crichton, R. R., & Bryce, C. F. A. (1973) *Biochem. J. 133*, 289–299.

Dixon, H. B., & Perham, R. N. (1968) *Biochem. J. 109*, 312–314.

Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L., & Yariv, J. (1984) *Philos. Trans. R. Soc. London, B 304*, 551–565.

Gerl, M., & Jaenicke, R. (1987a) *Biol. Chem. Hoppe-Seyler 368*, 387–396.

Gerl, M., & Jaenicke, R. (1987b) *Eur. Biophys. J. 15*, 103–109.

Gibbons, J., & Perham, R. N. (1970) *Biochem. J. 116*, 843–849.

Habeeb, A. F. S. A. (1966) *Anal. Biochem. 14*, 328–336.

Harrison, P. M., & Gregory, D. W. (1968) *Nature (London) 220*, 578–580.

Jaenicke, R. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 395–413.

Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.

Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp 68–77, Holden-Day, San Francisco.

Puigserver, A., & Desnuelle, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2442–2445.

Rice, D. W., Ford, G. C., White, J. L., Smith, J. M., & Harrison, P. M. (1983) *Adv. Inorg. Biochem. 5*, 39–50.

Smith-Johannsen, H., & Drysdale, J. W. (1969) *Biochim. Biophys. Acta 194*, 43–49.

Stefanini, S., Finazzi-Agrô, A., Chiancone, E., & Antonini, E. (1979) *FEBS Lett. 100*, 296–300.

Stefanini, S., Chiancone, E., Arosio, P., Finazzi-Agrô, A., & Antonini, E. (1982) *Biochemistry 21*, 2293–2299.

Stefanini, S., Vecchini, P., & Chiancone, E. (1987) *Biochemistry 26*, 1831–1837.

Wetz, K., & Crichton, R. R. (1976) *Eur. J. Biochem. 61*, 545–550.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Appendix: Self-Assembly Intermediates of Horse Spleen Apoferritin Considered in Relation to a Structural Model

Apoferritin assembly intermediates and the pathway they follow in forming complete tetracosamers can conveniently be visualized in a schematic form by use of a cutout model (Smith et al., unpublished results), Figure 6, based on the three-dimensional structure of horse spleen apoferritin (Banyard et al., 1978; Rice et al., 1983; Ford et al., 1984; White et al., unpublished results). Analysis of the structure shows it to be composed of twelve pairs of antiparallel subunits, each pair lying approximately on one of the twelve rhomb faces of a rhombic dodecahedron with subunit axes nearly parallel to one set of opposite rhomb edges (Clegg et al., 1980a,b). These subunit pairs have been suggested previously as likely assembly intermediates because they provide a long surface for intersubunit contact (Banyard et al., 1978), with interactions between neighboring A helices and L loops mainly involving apolar side chains, although hydrophilic interactions are also found (Ford et al., 1984). We propose that these account for much of the stable dimer population seen in the experiments described above and in Gerl and Jaenicke (1987a,b). In the native apoferritin structure rhomb edges are of two types, those lying parallel being equivalent to one another. The nonequivalent edges of the subunit pair rhombs coalesce as the whole quaternary structure comes together and the rhombs arrange themselves with their acute angles meeting at fourfold apices and obtuse angles at threefold apices. Simple inspection of the rhombic dodecahedral arrangement suggests possible assembly intermediates, as shown in Figure 6.

Figure 6a shows how the structure might assemble entirely from dimers (M_2) via tetramers (M_4) and hexamers (M_6). Two such hexamers could then join to form a dodecamer and

two dodecamers combine to give the complete tetracosameric molecule. This was the assembly route originally envisaged in Banyard et al. (1978), with the intermediate hexamer being a trimer of dimers. However, the experimental evidence for trimeric intermediates suggests the alternative formation of hexamers as dimers of trimers, Figure 6b. As can be seen, the two hexameric intermediates of (a) and (b) are not identical, in contrast to the two dodecamers formed from them, which are. In the pathway of Figure 6a, the monomer would be considered to be unstable, whereas the hexamer might be expected to be a stable intermediate. However, the experimental conditions examined in the present study and in Gerl and Jaenicke (1987a,b) lead to the pathway

$$24\mathcal{M}_1 \rightarrow 24M_1 \rightleftharpoons 8M_2 + 8M_1 \rightleftharpoons 8M_3 \rightleftharpoons (4M_6) \rightleftharpoons 2M_{12} \rightleftharpoons M_{24}$$

in which both monomers and dimers participate in trimer formation, and the hexamer of Figure 6b is only a transient intermediate. Consideration of Figure 6b and of intersubunit interactions within the actual native structure provides a rationale for this scheme.

The proposed trimer could form by combining the proposed dimer with a monomer as indicated in Figure 6b. There are extensive interactions along the rhomb interface, mostly apolar in character, and involving two different subunit pairs. These are in one case between the two hydrophobic E helices (clustered around fourfold axes in the native structure) and between the D helix of the "added" subunit and the DE and AB turns and the ends of A, B, and D helices from one subunit of the original dimer. The second subunit of this dimer makes interactions with the third protomer mainly involving the ends of C and D helices and the N-terminal tail. Details of these interactions will be described elsewhere (White et al., unpublished results). The same interface is of course found in the tetramer M_4 of the alternative pathway and, twice, in the hexamer M_6 of Figure 6a. However, stable tetramers do not seem to be an important intermediate experimentally. The observed transient nature of observed hexamers might be accounted for by the pathway, Figure 6b, where the hexamer formed by two M_3 trimers has two unused sets of rhomb dimer interactions both of which are completed in forming the dodecamer. In this step two of the eight hydrophilic threefold channels and one of the six hydrophobic channels are generated in each dodecamer. In the final rate-determining step of the assembly process, the two half-molecules must be exactly positioned by interacting at their eight free rhomb edges. In so doing the remaining four hydrophilic and four hydrophobic channels are formed.

Other assembly pathways may be constructed by examination of the model. For example, a symmetrical trimer intermediate could be formed from threefold axis related subunits, but in this case the dimer and dodecamer intermediates might be expected to be less stable than in our chosen pathway because the long twofold contacts at the centers of rhomb faces would be left exposed. Another possibility is the association of monomers or dimers around fourfold axes to give symmetrical tetramers or octamers. However, although this octamer could be imagined as a nucleation center on which dimers or other intermediates could be added [as outlined in Ford et al. (1974)], it is not possible to complete the shell by confluence of three such octamers for geometrical reasons (they would contain common subunit pairs).

Combination of the various cross-linking and chemical modification methods yields a sequential reaction scheme favoring the dimer, trimer, and dodecamer as well-populated intermediates, which can be explained in relation to the known structure as in Figure 6b.

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REFERENCES

Banyard, S. H., Stammers, D. K., & Harrison, P. M. (1978) *Nature (London) 271*, 282–284.

Clegg, G. A., Fitton, J. E., Harrison, P. M., & Treffry, A. (1980a) *Prog. Biophys. Mol. Biol. 36*, 53–86.

Clegg, G. A., Standfield, R. F. D., Bourne, P. E., & Harrison, P. M. (1980b) *Nature (London) 288*, 298–300.

Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L., & Yariv, J. (1984) *Philos. Trans. R. Soc. London, B 304*, 551–565.

Gerl, M., & Jaenicke, R. (1987a) *Biol. Chem. Hoppe-Seyler 368*, 387–396.

Gerl, M., & Jaenicke, R. (1987b) *Eur. Biophys. J. 15*, 103–109.

Rice, D. W., Ford, G. C., White, J. L., Smith, J. M., & Harrison, P. M. (1983) *Adv. Inorg. Biochem. 5*, 39–50.