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Subunit Interactions in Horse Spleen Apoferritin

DISSOCIATION BY EXTREMES OF pH

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1. The dissociation of horse spleen apoferritin as a function of pH was analysed by sedimentation-velocity techniques. The oligomer is stable in the range pH 2.8–10.6. Between pH 2.8 and 1.6 and 10.6 and 13.0 both oligomer and subunits can be detected. At pH values between 1.6 and 1.0 the subunit is the only species observed, although below pH 1.0 aggregation of the subunits to a particle sedimenting much faster than the oligomer occurs. 2. When apoferritin is first dissociated into subunits at low pH values and then dialysed into buffers of pH 1.5–5.0, the subunit reassociates to oligomer in the pH range 3.1–4.3. 3. U.v.-difference spectroscopy was used to study conformational changes occurring during the dissociation process. The difference spectrum in acid can be accounted for by the transfer of four to five tyrosine residues/subunit from the interior of the protein into the solvent. This process is reversed on reassociation, but shows the same hysteresis as found by sedimentation techniques. The difference spectrum in alkali is more complex, but is consistent with the deprotonation of tyrosine residues, which appear to have rather high p*K* values. 4. In addition to the involvement of tyrosine residues in the conformational change at low pH values, spectral evidence is presented that one tryptophan residue/subunit also changes its environment before dissociation and subsequent to reassociation. 5. Analysis of the dissociation and reassociation of apoferritin at low pH values suggests that this is a co-operative process involving protonation and deprotonation of at least two carboxyl functions of rather low intrinsic p*K*. The dissociation at alkaline pH values does not appear to be co-operative. 6. Of the five tyrosine residues/subunit only one can be nitrated with tetranitromethane. Guanidination of lysine residues results in the modification of seven out of a total of nine residues/subunit. Nine out of the ten arginine residues/subunit react with cyclohexanedione.

The physiological function of ferritin is to serve as a means of storing iron within the cell in a soluble form in which excess of iron may be deposited and from which iron can be mobilized as and when required. The structure of ferritin is well suited to such a functional role, consisting of a protein shell of 23–25 apoferritin subunits surrounding a micellar core of ferric hydroxyphosphate which contains from zero to 4500 iron atoms (reviewed, for example, by Harrison, 1964; Crichton, 1971*a*, 1973). The stability of the quaternary structure of apoferritin is clearly important in determining the mechanism by which iron is taken up by and mobilized from ferritin. It has been shown (Crichton *et al.*, 1973*b*; Jaenicke & Bartman, 1972) that even at concentrations as low as 0.5 μg/ml apoferritin is not dissociated into subunits. This does not, however, exclude the possibility that an equilibrium between oligomer and subunits exists, which lies well in the direction of the oligomer.

Apoferritin can be reversibly dissociated by a number of protein denaturants such as sodium dodecyl sulphate (Hofmann & Harrison, 1963; Smith-Johannsen & Drysdale, 1969; Crichton & Bryce, 1970) and guanidine hydrochloride (Bryce & Crichton, 1971; Björk & Fish, 1971), although this latter process has been recently reported to be strongly pH-dependent (Listowsky *et al.*, 1972). Treatment with 8–10M-urea does not cause apoferritin to dissociate (Hofmann & Harrison, 1963). It was reported by Harrison & Gregory (1968) that exposure of apoferritin to 67% (v/v) acetic acid, a procedure commonly employed for dissociation of viral coat proteins (Fraenkel-Conrat, 1957), resulted in stable subunit preparations at pH 3.0 which could be reassociated to oligomer at pH 5.0. Such subunit preparations were used by us to determine the polypeptide-chain molecular weight of apoferritin (Bryce & Crichton, 1971). We had also, in the course of an analysis of the susceptibility of ferritin and apoferritin to proteolysis (Crichton, 1971*b*), predicted and found evidence from circular dichroism for a conformational change in apoferritin below pH 3.0

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which was consistent with loss of some 15% of the helical structure, and involved a rather large change in the environment of aromatic amino acid residues (Wood & Crichton, 1971). Finally, in the course of an evaluation of the oligomer molecular weight we had observed that apoferritin at pH3.0 was not at all dissociated into subunits (R. R. Crichton & C. F. A. Bryce, unpublished work). Thus it appeared likely that apoferritin could be reversibly dissociated at low pH values and that a significant hysteresis occurred in the reassociation of the subunits to oligomer.

We present here an analysis by sedimentation techniques and u.v.-difference spectroscopy of the reversible dissociation of horse spleen apoferritin into subunits by extremes of pH and confirmation that the conformational change occurring on dissociation involves perturbation of the environment of tyrosine and tryptophan residues of the polypeptide chain. The co-operativity of the process has also been studied, and the $pK_{App.}$ of some of the groups involved in these conformation changes have been determined. The hysteresis observed in reassociation from low pH values enables us to prepare stable oligomers and stable subunits under identical conditions of pH and ionic strength, depending on whether the sample has been exposed to low pH or not. The reactivity of tyrosine, lysine and arginine residues towards specific reagents has been analysed to obtain information about the possible involvement of these residues in subunit–subunit interactions.

Experimental

Materials

Ferritin was prepared from horse spleen by the method of Granick (1943) as modified by Crichton *et al.* (1973*a*), or was purchased from Schwartz/Mann, Orangeburg, N.Y., U.S.A. Apoferritin was prepared by using thioglycollic acid (Crichton, 1973). Tetranitromethane was a product of Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; cyclohexane-1,2-dione was from Fluka A.G., Buchs, Switzerland; *O*-methylisourea was from Serva, Heidelberg, Germany; and thioglycollic acid was from British Drug Houses, Poole, Dorset, U.K. Sephadex was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. All other chemicals were analytical grade, or the purest grade available.

Methods

Preparation of samples for analysis. Samples of apoferritin for sedimentation-velocity studies, gel filtration or difference spectroscopy were prepared either by dissolving freeze-dried apoferritin, or by dialysing apoferritin solutions at the appropriate concentration into buffer of the required pH values and ionic strength. Usually 1ml of apoferritin solution was dialysed with stirring against 250ml of buffer at room temperature for 24h before analysis. For studies of the effect of pH on oligomer dissociation either 'Universal buffer' or 0.01M-glycine–HCl and glycine–sodium acetate buffers were used. Universal buffer was essentially a combination of Theorell and Stenhagen's citrate–phosphate buffer and Sörenson's glycine buffers I and II with a buffering capacity in the range 1.0–13.0 (Bates, 1968). The buffer was prepared from a stock solution containing 0.2M-citric acid, 0.2M-phosphoric acid, 0.2M-boric acid, 0.2M-glycine and 0.2M-NaCl; 50ml of stock solution was adjusted to the required pH value with 0.2–0.5M-NaOH or 0.2M-HCl and then made up to 250ml. The pH was then redetermined and adjusted if necessary. A Radiometer pH-meter, model 25, with scale expander (Radiometer, Copenhagen, Denmark) was used.

For reassociation studies at low pH values apoferritin was first dissociated either by treatment with 67% (v/v) acetic acid at 0°C for 1h, or else by dialysis against 0.01M-glycine–HCl buffer, pH1.50, for 24h at 25°C. The protein solution was then dialysed for 24h against two changes of either glycine or Universal buffer of pH1.6–4.5 at 25°C. Reassociation from high pH values was done by dialysis of apoferritin samples into Universal buffer, pH12.0, for 24h at 25°C, followed by dialysis into Universal buffer, pH12.0–10.0, for a further 24h at 25°C before analysis.

Sedimentation-velocity experiments. These were done with a Spinco model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, Calif., U.S.A.) equipped with an electronic speed-control unit, regulated temperature unit and a split-beam photoelectric scanner multiplex assembly. Sedimentation patterns were monitored at 280nm, or in a few cases at 230nm, in an AnG-Ti rotor at 116000*g* and 20°C. Sedimentation coefficients were corrected to the standard $s_{20,w}$ values as described by Schachman (1959). No correction for protein concentration was made; since in almost all cases a concentration of 0.5–1mg/ml was used the effect of protein concentration would not be expected to be great.

Gel filtration. Gel filtration of apoferritin samples (containing 5–10mg/ml) was done on columns (1.5cm×75cm) of Sephadex G-75, equilibrated with Universal buffer at the appropriate pH value. Modified apoferritins were analysed on a similar Sephadex G-75 column in 0.1M-sodium borate buffer, pH7.8. The columns were eluted at a flow rate of 20ml/h and 2ml fractions were collected in an LKB Ultra-Rac (LKB Produkter, Stockholm, Sweden). The absorbance of the effluent was measured at 230nm and 280nm. Blue Dextran [Pharmacia (G.B.) Ltd.], myoglobin (Boehringer, Mannheim, Germany) and tryptophan were used as reference materials.

Difference spectra. For difference spectroscopy a Cary 16 spectrophotometer (Cary Instruments, Monrovia, Calif., U.S.A.) equipped with a Cary 1626 recorder interface and a Honeywell Elektronik 194 recorder (Honeywell Controls, Newhouse, Lanarkshire, U.K.) was used. Solutions of apoferritin, prepared as described above for the sample and reference cells, were made from the same stock apoferritin solution and had absorbances at 280nm of 0.5–1.5. As a routine they were clarified before spectroscopy by centrifugation for 4min in a Beckmann Microfuge 52. Sufficient instrument warm-up time was allowed to eliminate the danger of amplifier drift during measurements. Baseline adjustment with buffer blanks for the range 200–400nm was performed before the protein spectra were determined. Slit-widths were never in excess of 0.8mm. For all experiments a matched pair of protein solutions of identical concentration was used. The effect of light-scattering was corrected by the method of Winder & Gent (1971) from the slope of the curve obtained in a plot of log E against log λ in the range 400–310nm. For the ionization of a tyrosine residue (Mihalyi, 1968) a molar absorption coefficient, ϵ_{295}, of 2381 litre·mol^{-1}·cm^{-1} was assumed. For the change in molar absorption produced by the transfer of a tryptophan chromophore from the interior of a protein into water, and for the perturbation of a tyrosine residue, $\Delta\epsilon_{max.}$ of 1600 (Donovan, 1969) and $\Delta\epsilon_{287}$ of 551 (Herskovits & Sorenson, 1968) were used respectively.

Chemical modification of apoferritin. Nitration of tyrosine residues was carried out by the method of Sokolovsky *et al.* (1966). Apoferritin (1–8mg/ml) in 0.05M-Tris–HCl buffer, pH8.0, was treated with a 0.84M-tetranitromethane solution in 95% (v/v) ethanol. The reagent was added in several 5μl portions with stirring. For studies of the time-course of the reaction a Cary model 16 spectrophotometer was used and the reaction course monitored at 428nm. Reagent was added to both sample and reference solutions. The modified protein was separated from excess of reagent on a column (30cm×2.5cm) of Sephadex G-25 eluted with 0.1M-NH_4HCO_3. A flow rate of 20ml/h was used and 2ml fractions were collected in an LKB Radirac fraction collector.

Guanidination of lysine residues was performed by the method of Tu *et al.* (1971). Apoferritin (50mg) was dissolved in 20ml of 0.6M-*O*-methylisourea and and pH adjusted to 10.5 with NaOH. The reaction proceeded for 3 days at room temperature (22°C) and the sample was then extensively dialysed for several days against 0.01M-sodium borate buffer, pH7.4. Chromatography on a column (30cm×2.5cm) of Sephadex G-25 in 0.1M-NH_4HCO_3 to remove the residual reagent was carried out as described above.

Arginine residues were converted into N^5-(4-oxo-1,3-diazospiro-4,4-non-2-ylidene)-L-ornithine by the procedure of Toi *et al.* (1967). Apoferritin (20mg) was dissolved in 2ml of 0.1M-triethylamine, adjusted to pH10.9 and treated with 10mg of cyclohexane-1,2-dione dissolved in 0.5ml of 0.1M-triethylamine, pH10.9, at room temperature for 24h. The reagent was removed by dialysis against 0.1M-triethylamine, pH10.0, and the pH was slowly decreased over a few days to a value of 7.4, by using 0.1M-sodium borate buffers. This procedure was used because when the reaction mixture was dialysed directly into buffer at pH7.4 the protein was precipitated.

Estimation of the degree of modification. (i) Tyrosine. The extent of nitration was estimated by amino acid analysis of the modified protein after acid hydrolysis. Nitrotyrosine was eluted from the amino acid analyser after phenylalanine. The colour factor was determined with an authentic sample of nitrotyrosine.

(ii) Lysine. The extent of modification of lysine after guanidination was determined by amino acid analysis from the decrease in the amount of lysine in the modified protein compared with the control and from the amount of homoarginine produced. Homoarginine was eluted after arginine and was estimated by using the same colour factor as for arginine. Homoarginine is reported to undergo little or no destruction on acid hydrolysis (Kimmel, 1967).

(iii) Arginine. Amino acid analysis of the modified protein was used to determine the extent of modification of the arginine residues by cyclohexanedione. The decrease in the arginine peak gave a measure of the degree of modification. To establish whether the modified arginine derivative could be identified directly by amino acid analysis, as described by Toi *et al.* (1967), we modified a sample of arginine under the same conditions as for the protein and subjected the products to amino acid analysis. The resulting elution profile on the short column of the analyser gave multiple peaks, including a peak of ornithine and of the putative derivative described by Toi *et al.* (1967). On account of the complexity of this elution profile, we made no further attempts to analyse the modification reaction in this way.

Spectrophotometric titration of nitrated apoferritin. A sample of nitrated tyrosine (3.2mg/ml) was adjusted in increments of 0.1–0.2 pH unit from pH6.5 to pH11.3 and the absorbance at 428nm measured. The increase in absorbance was then expressed as a function of pH.

Amino acid analysis. Amino acid analyses were carried out on protein samples containing 10–50nmol of apoferritin after hydrolysis with 6M-HCl *in vacuo* for 16h at 110°C. Bio-Cal (Bio-Cal Instruments, München-Gräfelfing, Germany), Locarte (Locarte Co., London W.7, U.K.) and Jeolco (Jeolco, London N.3, U.K.) analysers were used, with either single or two column operation and automatic sample loading. The analysis of the products obtained from treatment

of arginine with cyclohexanedione was done on the basic column of the Jeolco analyser.

Results

Sedimentation-velocity experiments

The effect of pH on the dissociation of apoferritin was examined by sedimentation-velocity experiments in Universal buffer at pH values between 1 and 13. Between pH 2.8 and 10.6 only one component was observed, with an $s_{20,w}$ value of 15.6–17.8S. At pH 4.5 (close to the isoelectric point) some protein was precipitated, but enough remained in solution for an estimation of the $s_{20,w}$ value to be made. As shown in Fig. 1, below pH 2.8 a slower-sedimenting component was observed, which was assumed to be subunit, since it had an $s_{20,w}$ value of 2.5–3.8 S. The percentage of slow-moving component increased steadily from pH 2.8 to 1.6, at which point only the slow-moving component was observed. Below pH 1.2, in addition to subunit, increasing amounts of protein were present as a high-molecular-weight aggregate, presumed to be denatured protein, which sedimented very much faster than the oligomer. Fig. 1 also shows the effect of dissociation of apoferritin in 67% (v/v) acetic acid (pH 1.63) followed by dialysis into Universal buffers of pH 1.5–5.0. Above pH 3.4 the protein was precipitated and no further results could be obtained. However, with dilute glycine buffers the protein remained in solution over the complete range of pH both for dissociation and for reassociation as shown in Fig. 2. The same profile was obtained on reassociation for protein that had been dissociated with acetic acid, or with glycine buffer of pH 1.5.

Dissociation of apoferritin at high pH values occurs above pH 10.6 (Fig. 3). At pH 13.0, although a slow component was observed, its $s_{20,w}$ value was found to be 0.25 S, which suggests that at this pH value hydrolysis of peptide bonds is taking place. To confirm this gel chromatography on Sephadex G-75 columns was carried out, and we established that above pH 12.4 products of alkaline hydrolysis were observed. Thus a study of the reassociation of apoferritin was undertaken from pH 12.0, where some 72% of the protein is present as subunit. The results are also shown in Fig. 3.

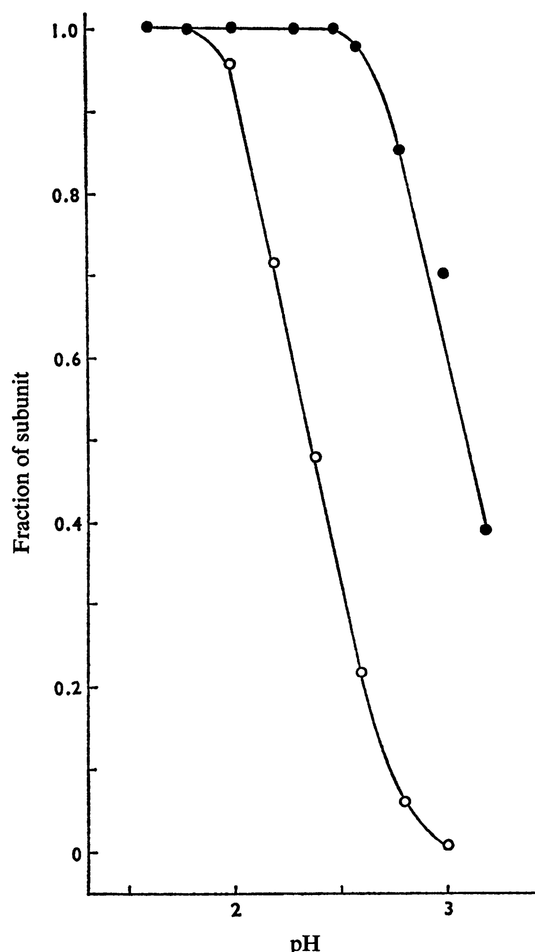

Fig. 1. *Dissociation and reassociation of apoferritin in Universal buffer, pH 1.6–4.0*

The results of sedimentation-velocity experiments are presented as fraction of protein present as subunit (determined from the E_{280} of the fast- and slow-moving components observed on the scanner traces). ○, Dissociation into buffer for 24h before analysis; ●, reassociation of samples first dissociated with acetic acid and then dialysed in buffer for 24h.

Difference spectroscopy

Acidification of a neutral solution of apoferritin results in a blue shift in the u.v. spectrum. A typical difference spectrum for an apoferritin solution at pH 3.06 relative to a solution at pH 1.63 is shown in Fig. 4. The magnitude of the difference maxima at 280nm and 287nm was proportional to protein concentration. Difference spectra for apoferritin in glycine buffers between pH 1.5 and 3.0 were collected with apoferritin at pH 1.5 as reference and in Fig. 2 the ratio of $\Delta\epsilon_{287}/\Delta\epsilon_{287,max.}$ is expressed as a function of pH. Fig. 2 also shows the same parameter for the reassociation of apoferritin from pH 1.50 in glycine buffers of pH 1.50–5.0; $\Delta\epsilon_{287,max.}$ was 2630. The difference spectra for the reassociation differ from those for dissociation above pH 4.0 (Fig. 5*a*) in having a long shoulder extending from 290nm to 320nm.

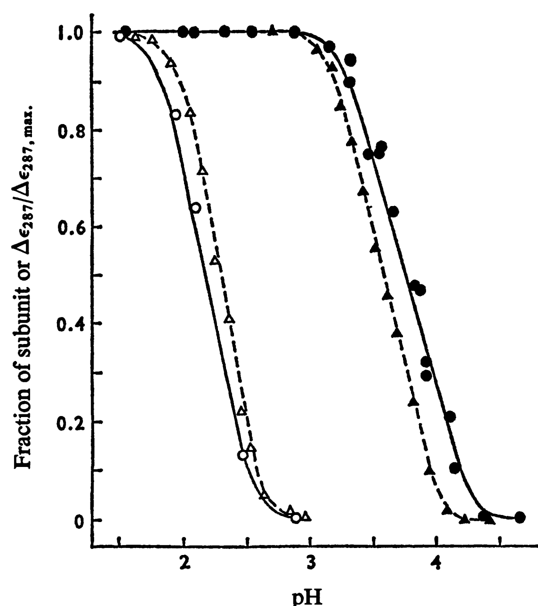

Fig. 2. *Dissociation and reassociation of apoferritin in glycine buffers, pH1.6–4.6*

○, Fraction of subunit for dissociation; ●, fraction of subunit for reassociation after dissociation in glycine buffer, pH 1.50, as described in the text. △, $\Delta\epsilon_{287}/\Delta\epsilon_{287,max.}$ for dissociation; ▲, $\Delta\epsilon_{287}/\Delta\epsilon_{287,max.}$ for reassociation after dissociation in glycine buffer at pH 1.50.

Such a spectrum would be expected from the perturbation of both tyrosine and tryptophan residues and Fig. 5(*b*) shows a perturbation difference spectrum calculated for five tyrosine residues and one tryptophan residue (Herskovits & Sorenson, 1968).

When apoferritin at pH 3.0 is used as reference and difference spectra are studied with apoferritin at pH 3.6–3.0 as sample, spectra are obtained which are characteristic of tryptophan perturbation. There is a sharp transition between pH 3.5 and 3.0 when $\Delta\epsilon_{294}$ is expressed as a function of pH (Fig. 6). The $\Delta\epsilon_{294,max.}$ value was 1600.

U.v.-difference spectroscopy was also used to study the effect of high pH values on apoferritin conformation. The difference spectrum in alkali had an absorption maximum at 294nm. The spectrophotometric titration of the tyrosine residues of the protein is shown in Fig. 3: $\Delta\epsilon_{294,max.}$ was 15040.

Reactivity of tyrosine, lysine and arginine

The reaction of apoferritin with tetranitromethane followed the time-course shown in Fig. 7, and the reaction was judged to be essentially complete in

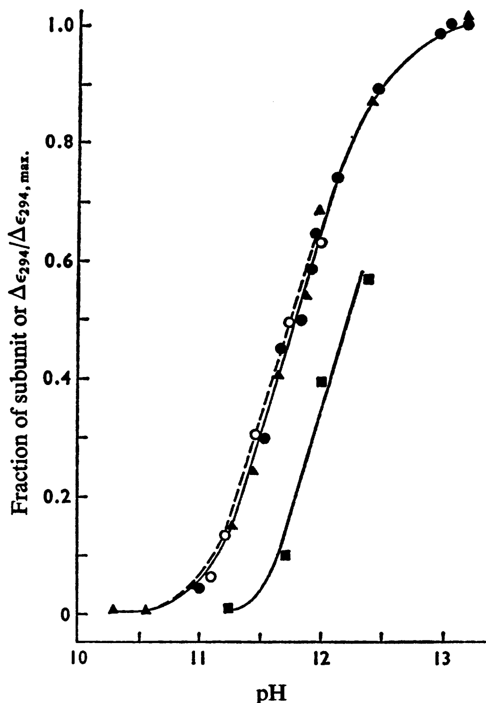

Fig. 3. *Dissociation and reassociation of apoferritin in Universal buffer, pH10.0–13.0*

●, Fraction of subunit on dissociation; ○, fraction of subunit on reassociation from pH 12.0 as described in the text. ▲, Spectrophotometric titration of tyrosine residues ($\Delta\epsilon_{294}/\Delta\epsilon_{294,max.}$); ■, fraction of subunit reported by Williams & Harrison (1968).

3h. From amino acid analysis (Table 1) we found 0.96 residue of nitrotyrosine and 3.85 residues of unmodified tyrosine/subunit. When the nitrotyrosine residue was titrated (Fig. 8) we found that it had an unusually high p*K* value, approx. 8.6.

The extent of guanidination of lysine residues in apoferritin was estimated both from the recovery of homoarginine and from the decrease in the amount of lysine (Table 1). Both values were in good agreement, indicating that seven lysine residues/subunit had been guanidinated.

The extent of modification of apoferritin with cyclohexanedione was estimated from the recovery of arginine on amino acid analysis (Table 1). We found that one arginine residue remains unmodified after this treatment.

Effect of modification on oligomer stability

The modified proteins were all analysed by sedimentation velocity and gel filtration. For the nitrated protein no slow-moving component was seen on

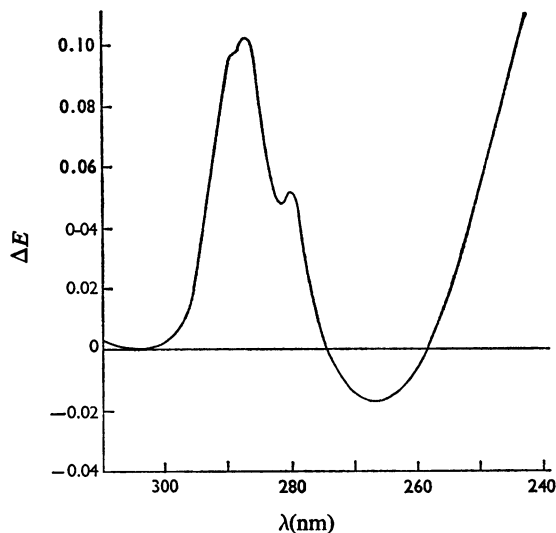

Fig. 4. *Difference spectrum for a solution of apoferritin* (0.069%, *w/v*) *at pH*3.06 *relative to a solution at pH*1.63

For details see the text.

analytical ultracentrifugation and the bulk of the protein had an apparent sedimentation coefficient of 16.9S. However, a continuous concentration-dependent association was observed and during the course of the centrifugation higher aggregates of the oligomer sedimented rapidly to the bottom of the cell, accounting for about 10% of the protein. In no case was apoferritin dissociated as a result of any of the chemical modifications that were carried out.

Discussion

Acid dissociation and reassociation

Apoferritin can be dissociated into subunits by treatment with acetic acid in the cold, and such subunit preparations are stable at pH3.0 but reassociate to oligomer at pH5.0 (Harrison & Gregory, 1968). Indeed, such subunit preparations can also be prepared by dialysis of apoferritin into buffer at pH1.5, and as shown in Fig. 2 these apoferritin samples also remain dissociated at pH3. In the experiments presented in Fig. 2, we dialysed apoferritin for 24h into buffer at pH3, and found only subunit. However, even in such samples, which had been maintained at pH3.0 for as long as 1 week, we could detect no

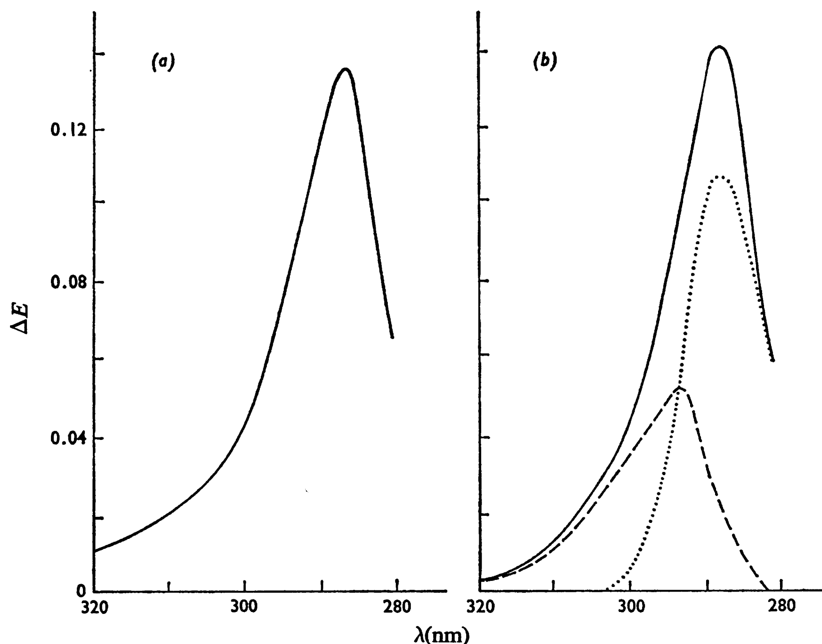

Fig. 5. *Perturbation difference spectra for apoferritin*

(*a*) Difference spectra for apoferritin (0.70mg/ml) at pH4.47 relative to the same solution at pH1.55; (*b*) theoretical spectrum (——) for perturbation of five tyrosine residues (······) and one tryptophan residue (----) calculated as described in the text for the appropriate concentration.

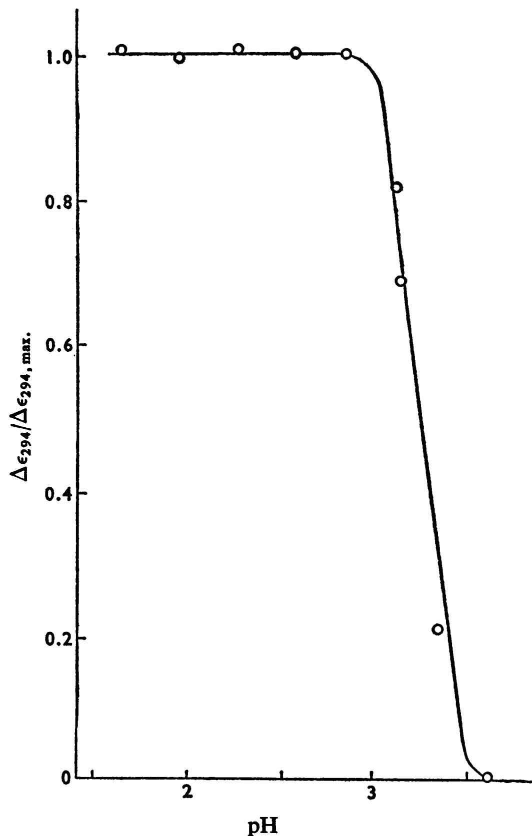

Fig. 6. *Perturbation of tryptophan residues in apoferritin before dissociation*

$\Delta\epsilon_{294}/\Delta\epsilon_{294,max.}$ is shown as a function of pH. Apoferritin (2.42mg/ml) at pH2.98 was the reference. For further details see the text.

oligomer. The acid dissociation is fully reversible (Fig. 2) and the cycle of dissociation–reassociation can be repeated without any change in the profiles shown in Fig. 2. It appears from a comparison of Figs. 1 and 2 that oligomer stability is affected by buffer composition and ionic strength, since the entire dissociation profile is shifted to lower pH values by 0.23 pH unit in the more dilute glycine buffers. Reassociation appears to proceed more readily in the Universal buffer; the effect here is much more marked and takes place 0.74 pH unit lower than in the dilute glycine buffer. The hysteresis observed in the Universal buffer is much less marked than in 10mM-glycine buffers (only 0.6 pH unit compared with 1.6 pH units).

The analysis of mixtures of several sedimenting components can be complicated by the Johnston–Ogston effect (Johnston & Ogston, 1946; Schachman,

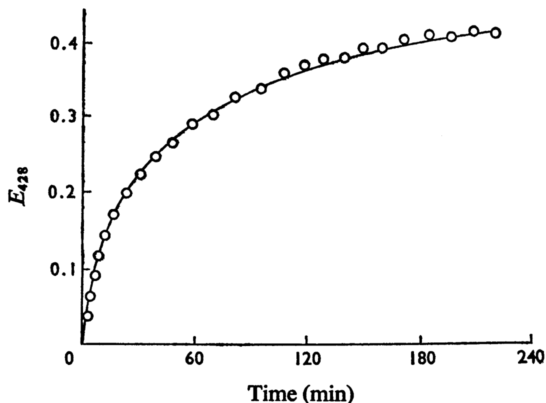

Fig. 7. *Time-course for nitration of apoferritin with tetranitromethane*

Conditions were as described in text. Protein concentration was 1.85mg/ml. E_{248} measures the formation of nitrotyrosine.

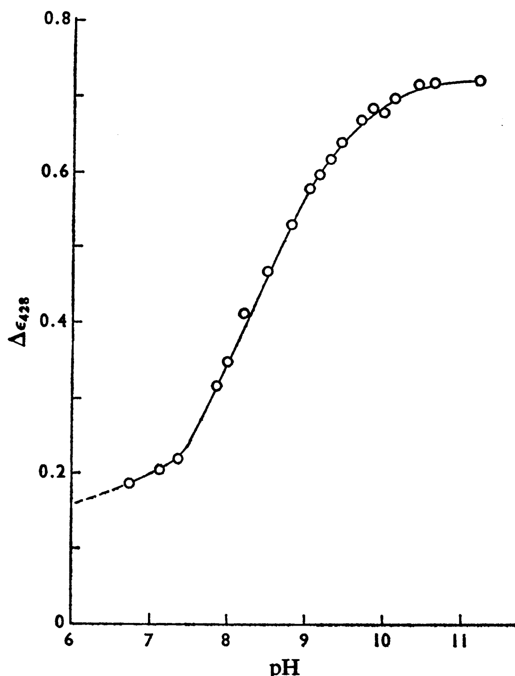

Fig. 8. *Titration of nitrated apoferritin from pH*6.8 *to* 11.2

Protein concentration was 1.58mg/ml. Conditions were as described in the text.

1959). However, in the present system, where the fast-moving component has an *s* value of 16–17S and the slow component an *s* value of 2–3S the

Table 1. *Amino acid composition of modified apoferritin*

The results are presented as residues of each amino acid/subunit, based on a molecular weight of 18500. The results are the mean of at least three separate determinations. The values for unmodified apoferritin are from Bryce & Crichton (1971). Abbreviations: Har, homoarginine; Tyr(3NO_2), 3-nitrotyrosine. *Italic* numerals indicate significant differences between modified and unmodified protein.

	Amino acid composition of apoferritin (residues/subunit)			
Amino acid	Unmodified	Modified with tetranitromethane	Modified with *O*-methylisourea	Modified with cyclohexanedione
Asx	17.3	17.7	17.7	17.9
Thr	5.5	5.6	5.6	5.5
Ser	9.0	8.5	8.8	8.8
Glx	23.9	23.8	23.9	24.0
Pro	2.8	1.6	2.6	2.9
Gly	9.9	10.0	9.9	9.8
Ala	14.0	13.7	13.9	13.8
Val	6.9	6.3	6.6	6.2
Met	2.8	2.9	2.3	2.2
Ile	3.5	3.6	2.9	2.9
Leu	25.0	25.0	24.9	24.9
Tyr	5.0	*3.92*	4.6	5.0
Phe	7.3	7.4	7.4	7.6
Tyr(3NO_2)		*0.96*		
His	5.8	5.4	5.7	5.6
Lys	8.7	8.2	*1.92*	Not determined
Arg	9.5	10.1	9.8	*0.86*
Orn				*0.87*
Har			*6.66*	

Johnston–Ogston effect would be negligible. From the results presented here, it might be thought that the dissociation–reassociation occurs with no intermediates between oligomer and subunit, so that at intermediate pH values such as between 2.8 and 1.6 in glycine buffers (Fig. 2) a steady state exists in which both oligomer and subunit are present at concentrations, that are dependent solely on the pH (the percentage of fast- and slow-moving component did not change with increasing time of dialysis). Under the conditions used here it would be impossible to distinguish in the slow-moving component between monomers, dimers etc. We have in fact found by gel filtration (Crichton, 1972; R. R. Crichton, unpublished work) that in the course of both dissociation and reassociation not only monomers but also dimers and tetramers are present.

Apoferritin is much more rapidly digested by pepsin at pH2.5 than at pH3.0, although the pattern of peptides produced does not alter (Crichton, 1971*b*). We proposed that this effect was due to a conformational change in the protein, and analysis by optical rotatory dispersion and circular dichroism showed that some 10–15% of the ordered secondary structure was lost between pH3.0 and 2.2 (Wood & Crichton, 1971). These investigations were done in 100mM-citrate buffers and are most directly comparable with the results shown in Fig. 1. It was also clear from the near-u.v. circular-dichroism results that considerable changes occurred between 270 and 295nm, and we concluded that tyrosine and/or tryptophan residues were involved in the conformation change, which is clearly the process of subunit dissociation.

The difference spectra at low pH values parallel the results obtained by sedimentation velocity very closely (Fig. 2), and it seems reasonable to assume that they reflect changes in the conformation of the apoferritin molecule that are a consequence of the subunit dissociation. On dissociation four to five tyrosine residues/subunit are transferred to a more hydrophilic environment. We cannot be sure whether all five tyrosines are involved, since values quoted in the literature for tyrosine perturbation vary considerably.

The effect of decreasing the pH further, by using the apoferritin subunit at pH1.5 as reference, was to decrease the blue shift, indicating that some refolding

or aggregation was taking place. This agrees with the results of sedimentation-velocity experiments described above.

The perturbation of tryptophan between pH 3.6 and 3.0 shown in Fig. 5 occurs before subunit dissociation. From the literature value of the $\Delta\epsilon_{294}$ for tryptophan perturbation we conclude that one tryptophan residue is transferred from the interior of the protein to the solvent. The only amino acid side chain that could be involved in such a pH-dependent transition with a pK of 3.25 would be a carboxyl group. The transition is very sharp and can perhaps best be explained in terms of an 'ionization explosion' (Scheraga, 1961) which manifests itself in an abnormal steepening of the titration curve in the region in which the carboxyl groups ionize. Such a titration anomaly has been attributed, for bovine serum albumin, to carboxyl–carboxyl dimer bonds (Loeb & Scheraga, 1956).

Interpretation of the difference spectra for reassociation of apoferritin from low pH values is rather difficult. Below pH 4.0 only tyrosine residues seem to be involved, and the difference in spectral maximum at 287 nm follows the reassociation of subunit to oligomer quite closely. However, the shoulder from 290 to 320 nm (Fig. 5*a*) indicates that the tryptophan residue that was perturbed before dissociation returns to the interior of the protein, and this view is supported by the good agreement between Fig. 5(*a*) and the theoretical spectrum shown in Fig. 5(*b*), calculated for perturbation of five tyrosine residues and one tryptophan residue (Herskovits & Sorenson, 1968).

By using the equations derived by Herskovits & Sorenson (1968) we could establish that the single perturbed tryptophan residue was transferred into the interior of the protein in the pH range 4.0–4.5.

Dissociation at alkaline pH values

Williams & Harrison (1968) have shown that partial disaggregation of apoferritin occurs at high pH values. The results from their paper are included for comparison in Fig. 3, and it is apparent that dissociation under their conditions (namely in 20 mM-sodium borate buffer) occurs at about 0.44 pH unit to the alkaline side of that found by us. This again implies, in parallel to our findings at low pH values (Figs. 1 and 2) that subunit dissociation is facilitated in the more concentrated Universal buffer. The analysis of subunit reassociation from high-pH subunits was not possible, as discussed above, because of degradation of apoferritin to lower-molecular-weight materials. We tried to reassociate apoferritin from pH 13.0 in buffers of lower pH, but were unable to obtain satisfactory results. The reassociation experiments were therefore carried out from pH 12.0, and Fig. 3 shows that reassociation from 70% subunit exactly parallels the results for dissociation; this process would imply a state of true thermodynamic equilibrium, unlike that seen on going from 100% acid subunits to oligomer at low pH.

The difference spectra in alkali showed one major absorption maximum at 294 nm and the change in molar absorption parallels the subunit dissociation exactly. From the $\Delta\epsilon_{294}$ we conclude that, at alkaline pH values, all five tyrosine residues are titrated together. As was found with the sedimentation study, reassociation was not possible from pH 13.0. Reassociation from pH 12.0 gave oligomer with the normal apoferritin spectrum at pH 7.0.

Co-operativity of conformational changes

We have discussed above the high degree of co-operativity in the perturbation of a single tryptophan residue/subunit just before the beginning of subunit dissociation. The conformational changes associated with subunit dissociation and reassociation at low pH values are associated with the protonation of one or more acidic groups, and in the pH range these must be carboxyl groups. If the equilibrium involves only one microscopic species, such as carboxylate ion and carboxyl, the apparent ionization constant, pK, can be calculated from the equation:

$$\mathrm{p}K = \mathrm{pH} - \log[\alpha/(1-\alpha)]$$

where α is the fraction of one species, and the value of pK is invariant with α within experimental error (Wetlaufer, 1962). However, when a graph of pK against α is constructed from the experimental results in Fig. 2, a linear relationship with zero gradient is not obtained, implying that more than two microscopic species are involved.

Since multiple equilibria appear to be involved we constructed graphs of $\log[\alpha/(1-\alpha)]$ versus pH (Hill plots) to distinguish between co-operative and non-co-operative effects. Least-squares fits of the results in Figs. 2 and 3 to a linear relationship between $\log[\alpha/(1-\alpha)]$ and pH gave the characteristic pH values (the pH value at which $\alpha = 0.5$) and slopes shown in Table 2. Within experimental error the fraction of subunit present shows the same pH-dependence as the $\Delta\epsilon_{287}$. The slopes, both for dissociation and reassociation in the acid pH range, are around 2, indicating that the titratable groups of apoferritin which stabilize subunit association and oligomer conformation are protonated in a co-operative manner.

We conclude tentatively from the Hill plots that dissociation and reassociation of apoferritin at low pH values involves the protonation of at least two carboxyl groups. The apparent pK value of these groups for dissociation is 2.16 and for reassociation 3.78. The value of 2.16 for dissociation is rather low

Table 2. *Hill-plot parameters for apoferritin dissociation and reassociation in glycine buffers*

Parameters were calculated from the results in Figs. 2 and 3 as described in the text, both from the sedimentation-velocity data (fraction of subunit) and from the difference-spectral data ($\Delta\epsilon_{287}$ or $\Delta\epsilon_{294}$).

	Intercept (pH)	Slope
Acid dissociation		
Fraction of subunit	2.16	2.12
$\Delta\epsilon_{287}$	2.12	2.07
Acid reassociation		
Fraction of subunit	3.77	1.96
$\Delta\epsilon_{287}$	3.72	1.70
Alkaline dissociation		
Fraction of subunit	11.82	1.05
$\Delta\epsilon_{294}$	11.80	1.05

for β- or γ-carboxyl groups of aspartyl or glutamyl side chains (4.1 and 4.5 respectively; Nozaki & Tanford, 1967) though not for an α-carboxyl group, and would suggest that those carboxyl groups are in a rather non-hydrophobic environment, possibly involved in salt linkages with lysine or arginine residues. On reassociation, the apparent p*K* is much closer to that expected for a normal carboxyl group. This is to be expected, since the dissociated protein is in a more open structure as evidenced by the environment of the tyrosine residues, and since the local constraints that restricted the protonation of these groups in the oligomer have been removed.

The alkaline dissociation process shows no cooperativity (Table 2): the slopes from the Hill plots are close to 1. All five tyrosine residues titrate with a $pK_{app.}$ of 11.8. The p*K* of tyrosine residues in proteins is a function of their environment, and usually exposed tyrosine residues would be expected to have a p*K* of about 9.6 (Tanford, 1962). However, if the tyrosine residues were 'buried' in a hydrophobic protein–protein interface, the p*K* would be increased. Thus, for example in ribonuclease three of the tyrosine residues have a p*K* of 13 (Tanford *et al.*, 1955).

Reactivity of tyrosine, lysine and arginine residues

We have not yet established whether nitration of apoferritin introduces a nitro group on only one tyrosine residue/subunit, or whether nitration of several tyrosine residues, occurs giving a total value of one nitro group/subunit. The reactivity of tyrosine residues reflects in part their degree of exposure to solvent and in part their local environment. Myers & Glatzer (1971) have observed that in subtilisin nitration of some tyrosine residues in non-polar environments within the protein takes place. This may be the case with apoferritin. This view is strengthened by the observation that the p*K* of the nitrotyrosine residue in apoferritin is abnormally high compared with the value of approx. 7 which is usually found (Sokolovsky *et al.*, 1966; Cuatrecasas *et al.*, 1968).

Conversion of protein amino groups into guanidino groups by *O*-methylisouronium salts seems to be highly selective. At pH 10.5 lysine residues are quantitatively converted into homoarginine with few if any side reactions, although some modification to the α-amino groups of the *N*-terminal amino acid has been reported (Kimmel, 1967). This would not be a problem in apoferritin, which has no free α-amino group (Suran, 1966). The guanidination of seven out of nine lysine residues does not affect the oligomer stability; both by gel filtration and by sedimentation velocity, no subunit was detected. The fact that two lysine residues do not react with *O*-methylisourea suggests that these lysine residues may either be buried inside the protein, or else have p*K* values that are abnormally high (the reaction with *O*-methylisourea was carried out at pH 10.5) perhaps due to salt linkage with carboxyl groups.

The reaction of cyclohexanedione at pH 11 with arginine generates N^5-(4-oxo-1,3-diaza-spiro-4,4-non-2-ylidene)-L-ornithine, which is reported to be stable to acid hydrolysis (Toi *et al.*, 1967). We were unable to determine this derivative by amino acid analysis, but could determine the amount of arginine remaining after modification (Table 1). It seems that one arginine residue is not modified under conditions (0.1 M-triethylamine, pH 10.9) where subunit dissociation does not occur. Again the modified protein was found to be present as oligomer only.

None of the modifications described above affect the catalytic function of apoferritin in oxidation of Fe^{2+} (Bryce & Crichton, 1973).

The fact that two lysine residues and one arginine residue/subunit are not modified by reagents which are specific for these two amino acids would imply that those three residues are either not accessible to the reagent, or else are in a local environment in which their reactivity is decreased. It is possible that they are 'buried' in the subunit–subunit interface, but such an interpretation must clearly be treated with caution.

Subunit–subunit interactions in apoferritin

From the results presented here we can summarize the changes in conformation that occur on dissociation of apoferritin by extremes of pH. At low pH values, the first transition to occur involves the transfer of one tryptophan residue/subunit from the interior of the protein to the solvent; accompanying this

transition one or more carboxyl groups of pK 3.29 is protonated. Subunit dissociation involves the transfer of four to five tyrosine residues/subunit to a more hydrophilic environment, most likely to the solvent, accompanied by protonation of at least two carboxyl groups of pK 2.16 (which might be in salt linkage with lysine or arginine residues). On re-association the entire sequence is reversed, the carboxyl groups are deprotonated with a pK of 3.86 and the tyrosine and tryptophan residues finally return to the interior of the oligomer. At high pH values all five tyrosine residues are deprotonated with a pK of 11.8 in the course of subunit dissociation. It remains to be established whether the two lysine residues and one arginine residue that are not modified at high pH values are buried at the subunit interface and whether they are involved in salt linkages with carboxyl functions.

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