Hydrogen Ion Interactions of Horse Spleen Ferritin and Apoferritin*

SUSAN T. SILK and ESTHER BRESLOW
From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021

The iron storage protein ferritin consists of a spherical protein shell containing 24 subunits surrounding a micelle of ferric hydroxyphosphate (1, 2). The subunits are most generally assumed to be identical with a molecular weight of 18,500 (2, 3), although recent studies suggest that some variation in subunit constitution may be present (4). Relatively little is known about the primary structure or conformation of the subunits. A high α helix content is known to be present (2, 5) and a few side chain modification studies have been reported (6-8). Optical activity studies (5, 9) suggest that the conformational differences between the two proteins are absent in apoferritin and possibly also the presence of anion binding sites in apoferritin that are occupied in ferritin by anions of the core. The difference between the isoelectric points of the two proteins in KCl has been interpreted to indicate the presence of approximately 2 phosphate residues per ferritin subunit which serve as cation binding sites and which are negatively charged at the isoelectric point in ferritin. Effects of KCl and NaCl on the two proteins indicate the presence of cation binding sites in ferritin that are absent in apoferritin, but suggest that differences between the two proteins below pH 7.5, are probably attributable directly or indirectly to the presence of phosphate residues at the surface of the ferritin core. Accordingly, we have studied the H+ ion titration properties of horse spleen ferritin and its derivative apoferritin, assessing with particular emphasis the relationship between the two proteins. In order to analyze the titration data, it was necessary to determine the amide composition of ferritin, most useful methods for assessing the environment and solvent accessibility of ionizable residues on proteins is by determining their reactivity to H+ ions (11, 12). H+ ion reactivity can also be a sensitive indicator of conformational changes (11-13) and therefore, in this instance, might be useful in further assessing conformational differences between ferritin and apoferritin which are directly or indirectly attributable to effects of the iron core. Accordingly, we have studied the H+ ion titration properties of horse spleen ferritin and its derivative apoferritin, assessing with particular emphasis the relationship between the two proteins. In order to analyze the titration data, it was necessary to determine the amide composition of ferritin, since this has not previously been reported. Interpretation of the H+ ion titration curves has additionally been aided here by selected salt-binding and side chain modification studies. The results indicate important differences between ferritin and its derivative apoferritin, which are directly or indirectly attributable to changes in the iron incorporation process (6). One of the most useful methods for assessing the environment and solvent accessibility of ionizable residues on proteins is by determining their reactivity to H+ ions (11, 12). H+ ion reactivity can also be a sensitive indicator of conformational changes (11-13) and therefore, in this instance, might be useful in further assessing conformational differences between ferritin and apoferritin which are directly or indirectly attributable to effects of the iron core. Accordingly, we have studied the H+ ion titration properties of horse spleen ferritin and its derivative apoferritin, assessing with particular emphasis the relationship between the two proteins. In order to analyze the titration data, it was also necessary to determine the amide composition of ferritin, since this has not previously been reported. Interpretation of the H+ ion titration curves has additionally been aided here by selected salt-binding and side chain modification studies. The results indicate important differences between ferritin and its derivative apoferritin, which are directly or indirectly attributable to changes in the iron incorporation process (6).

EXPERIMENTAL PROCEDURES

Protein Preparation—Horse spleen ferritin was prepared by the method of Granick (14) or the two and six times cadmium-recrystal-
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lized protein was purchased from Miles Laboratories. Prior to titration or other physicochemical studies, all cadmium-recrystallized ferritin solutions were dialyzed at 4°C for several days against 0.01 M EDTA in 0.01 M phosphate buffer, pH 7, and then exhaustively against 0.16 M KCl.

Apoferritin was prepared from cadmium-recrystallized ferritin which had first been exhaustively dialyzed against deionized water, using Method II of Granick and Michaelis (15) or the procedure of Bjork (16) omitting the Sepharose 6B column. Stock solutions of apoferritin were obtained by exhaustive dialysis against 0.16 M KCl at 4°C. The concentration of iron in apoferritin was determined by reaction with K3Fe(CN)6 and found to be less than 0.1 g atom of iron/subunit. On polyacrylamide gel electrophoresis at pH 8.5 in 5% gels, apoferritin showed the same percentage (2) of monomer (approximately 8%) and oligomers (approximately 15%) as its parent ferritin.

The concentrations of all protein stock solutions were determined in duplicate by micro-Kjeldahl nitrogen analysis following digestion in the presence of HgO, Na2SO3, and concentrated H2SO4 (17). The relationship between the per cent of nitrogen and protein weight was determined as 5.81 mg of apoferritin/mg of nitrogen using the amino acid analysis and amide content determined in these studies. Occasional ferritin solutions were standardized by Folin analysis (18) using a standard curve. All protein concentrations were determined with a ferritin that had been standardized by nitrogen analysis for the protein concentration of the fraction (11, 12). A batch procedure was also used to determine the pI of a 1% ferritin solution; successive additions of M B-1 resin were made and then removed until a constant pH was achieved. The pI of 1% ferritin in H2O was 4.58 ± 0.02; the pI of apoferritin of the same concentration was identical with that of ferritin within experimental error. For both proteins, isoelectric pH values identical with those obtained by ion exchange were also obtained by exhaustive dialysis against H2O.

H+ Ion Titrations—Continuous potentiometric titrations were conducted under N2 as previously described (26) with the modification that the entire titration assembly was housed in a polyethylene glove box which was pre-purged with N2 to further decrease contamination by CO2. A Radiometer model pHM 64 pH meter was used and was routinely standardized at pH 7 and checked with standard buffers at pH 4 and 10 to ensure accurate response over the pH range of the titration. Highest purity nitrogen gas was passed over an Ascarite bed and a solution of either 0.16 or 1 N KCl prior to passing through the titration solution and the glove box. Generally, 2 ml of protein solution at 9 to 10 mg/ml were titrated. Titrants were 0.1 N NaOH in 0.16 M KCl and 0.1 N HCl in 0.06 M KCl for titration at 0.16 ionic strength and 0.1 N NaOH in 1 N KCl and 0.05 N HCl in 0.93 M KCl for titration at ionic strength of 1. Titrations were always started at pH 4.5 to 5.0 with sufficient time (30 s to 3 min) allowed after each addition of acid or base for the pH to become constant except as noted. At the completion of each titration the response of the pH meter to standard buffers was rechecked; for titrations in which results were regarded as valid, standardization drifts during titration were less than 0.02 pH unit.

Spectrophotometric titrations of apoferritin at 0.10 and 1 ionic strength were performed in glycine/KCl buffer using methods previously described (13). Changes at 295 and 245 nm as a function of pH were recorded.

General Methods—All reagents were analytical grade and deionized water was used throughout. Circular dichroism studies were performed using a Cary 60 spectropolarimeter equipped with a model 6001 circular dichroism attachment.

RESULTS

Amide Content of Ferritin and Apoferritin—Two methods were used to estimate this value. (see “Experimental Procedures”). First, the amide content was estimated directly from the NH2 released on partial hydrolysis. Second, the carbonyl groups of the denatured protein in 7 M guanidine were modified by reaction with glycine and the excess glycine determined by amino acid analysis. Results are shown in Table I. Twenty-six non-amide side chain carbonyl groups are indicated by the partial hydrolysis data. This value is also well within experimental error of the value obtained by reaction with glycine even when potential contributions of the α-COOH to the glycine reaction are considered.

Circular Dichroism of Ferritin and Apoferritin—Fig. 1 shows the near-ultraviolet circular dichroism spectrum of apoferritin between pH 6 and 3 and the far-ultraviolet circular dichroism spectra of ferritin and apoferritin near neutral pH; the high absorbance of ferritin in the near-ultraviolet precludes obtaining near-ultraviolet ferritin spectra. Both the near- and far-ultraviolet ellipticity spectra of the chemically prepared apoferritin are very similar to the spectra reported elsewhere (27) for “natural” apoferritin (that isolated directly from horse spleen), while ellipticity differences between the chemically derived apoprotein and ferritin in the far-ultraviolet are comparable in magnitude to previously observed (5) differ-

1 The abbreviations used are: pI, isoelectric pH; pL, isoelectric pI.
Changes in the far-ultraviolet ellipticity spectra of ferritin with
lowering the pH, however, conformational changes occur in the
environment of apoferritin aromatic chromophores with
decreasing pH (as manifest by changes in near-ultraviolet
ellipticity) than of loss of α-helical content (as manifest by
changes in far-ultraviolet ellipticity) is in accord with the ob-
servation (29) that, at low pH, changes in near ultraviolet
absorbance precede apoferritin subunit dissociation.

Effect of Salts on the Isoionic pH of Ferritin and Apoferritin—In
water, the isoionic points of 1 to 2% preparations of
ferritin and apoferritin are 4.58 ± 0.02 (see “Experimental Pro-
cedure”). Both proteins are partially insoluble in the absence of
salt and become soluble at very low salt concentrations.
Since H\textsuperscript{+} titrations are performed in the presence of KCl, the
effect of KCl on the isoionic points of ferritin and apoferritin
was determined by noting the change of pH produced by addi-
tion of increasing KCl concentrations to 1.5 to 2.0% prepara-
tions of the isoionic proteins in H\textsubscript{2}O (Fig. 2); at least 10 min
was allowed after each addition for equilibrium to be obtained.
Increasing KCl produces a large increase in the isoionic pH
(p\textsubscript{I}) of apoferritin that is particularly marked at low KCl
concentrations. This effect is too large and in the wrong
direction to be attributed to effects of KCl on activity
coefficients in a pH region where carboxylic acids are the
dominant buffer. For example, in agreement with theory (30),
the pH of a solution of 5 \times 10\textsuperscript{-5} M acetic acid plus 5 \times 10\textsuperscript{-4} M
sodium acetate decreases from 4.795 in the absence of added
salt to 4.705 when sufficient KCl is added to make the solution
0.11 M in KCl. Additionally the change in p\textsubscript{I} is in the wrong
direction to be attributed to the increased protein solubility in
the presence of salt (11). Using traditional arguments (11, 31),
the increase in pH when KCl is added to apoferritin is therefore
tentatively attributed to binding of Cl\textsuperscript{−}.

By contrast with apoferritin, low concentrations of KCl
produce an initial decrease in the p\textsubscript{I} of ferritin which then
gradually increases as the KCl concentration is further in-
creased. The initial decrease in ferritin p\textsubscript{I} is sufficiently large
that it probably results only partially from salt effects on
activity coefficients (see above) and is suggestive of K\textsuperscript{+}
binding. The subsequent increase in p\textsubscript{I} at higher KCl concentra-
tions is attributable to Cl\textsuperscript{−} binding. K\textsuperscript{+} binding apparently
being stronger than Cl\textsuperscript{−} binding in this case. That K\textsuperscript{+} binding
to ferritin does occur is supported by the lesser effects of NaCl
than of KCl on the p\textsubscript{I} of ferritin (Fig. 2). NaCl and KCl should
produce similar changes in pH if their effect were only to alter
activity coefficients (31). The lesser effect of NaCl than of KCl
on ferritin (which was repeatedly observed with different salt
preparations) suggests that there are cation binding sites on
ferritin that exhibit cation selectivity; i.e. Na\textsuperscript{+} binds more
weakly than K\textsuperscript{+}. Interestingly, NaCl appears to reproducibly
produce slightly smaller pH increases than does KCl with apo-
ferritin; while no simple explanation is available, the fact that
NaCl does not generate a larger pH increase than does KCl
with apoferritin suggests that apoferritin lacks the cation bind-
ing sites found on ferritin. In sum, the data strongly suggest the
presence of anion binding sites on apoferritin the strongest of
which are either unavailable in ferritin or overshadowed by the
presence of cation binding sites.

**Titration of Apoferritin in 0.16 M KCl**—Apoferritin titrations
(see “Experimental Procedures”) were initiated at pH 5, and

| TABLE I  |
|-----------|
| Amide content of ferritin and apoferritin |
| Content | Residues per subunit |
|----------|----------------------|
| Total aspartic + glutamic \* | 41 |
| NH\textsubscript{4} \* | 15 ± 0.5 |
| β,γ-Carboxyls \* | 26 ± 0.5 |
| Glycine (before carboxyl modification) | 9.9 |
| Glycine (after carboxyl modification) | 35.6 ± 1.3 |
| Total modifiable carboxyls | 25.7 ± 1.3 |

\* Obtained from amino acid analysis.
\* Obtained from partial hydrolysis. Values are recorrected for the change in per cent nitrogen in the protein resulting from the amide content.

\* Assumes that all the amides are on side chain carboxyl groups.
Effects of KCl and NaCl are compared using the same protein preparation. The initial pH in the absence of salt was 4.58. Effects of KCl and NaCl are compared using the same protein preparation.

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Fig. 2. Semilogarithm plot of the effect of KCl and NaCl on the pH at the isoionic point of 1.5 to 2.0% solutions of ferritin and apoferritin. ○, ferritin + KCl; □, ferritin + NaCl; ■, apoferritin + KCl; △, apoferritin + NaCl. The initial pH in the absence of salt was 4.58. Effects of KCl and NaCl are compared using the same protein preparation.

Fig. 3. Titration studies of apoferritin, 25°, 0.16 M KCl. $h$ is the number of protons bound or dissociated, per subunit, setting $h = 0$ at the isoionic pH (4.96) in 0.16 M KCl. ○, forward-titration data from pH 4.96 with acid or base; ●, rapid back-titration from pH 3; △, rapid back-titration from pH 2. Different samples behaved differently between pH 9 and 11; back-titration from pH 11 is shown (×) for sample showing the greatest number of dissociated protons in this region on forward-titration. Solid line is theoretical line described by parameters in Table III. Inset shows the change in molar extinction at 245 and 295 nm observed on spectrophotometric titration. ○, 245 nm forward-titration with base; ●, 245 nm reverse titration from pH 13; △, 295 nm forward-titration with base; ▼, reverse titration from pH 13.

The pH was adjusted continuously in small increments with either acid or base and then returned in similar fashion to the starting pH. With the exception that a small degree of irreversibility was noted on back-titration from pH 11, titrations were reversible and pH equilibrium was rapidly achieved in those studies in which the pH did not extend into the region of circular dichroism change. Where titrations extended into denaturing pH regions, the extent of irreversibility paralleled the extent of conformational change evident from CD studies. Thus, titration to and from pH 8 was rapidly reversible with no significant pH drifts noted, back-titration from pH 3 showed a small degree of irreversibility on the titration time scale and that from pH 2 showed marked irreversibility; regions of irreversibility were accompanied by drifts in pH that suggested a time-dependent return to the native structure and protein that had been exposed to denaturing conditions became markedly insoluble on back-titration to pH 5. It is relevant to note, however, that protein back-titrated to pH 5 from pH 8 or lowered in pH to 4, also showed some insolubility, despite the rapid reversibility of the titration curves and the lack of CD changes in these pH regions, suggesting that some aggregation had occurred without effect on $H^+$ ion equilibria.

Fig. 3 is a composite titration curve of all preparations of apoferritin studied. The results presented are largely of titration from pH 5 to 11 and from pH 5 to 2. Also shown are
back-titration data from pH 3, pH 2, and pH 11. Not shown are the back-titration data from pH 8 (identical with the forward-titration). The back titration data from pH 2 must be interpreted with caution since they were obtained by very rapid titration to prevent significant conversion of denatured to native protein and equilibrium of H+ with the very heavy precipitate that is present under these conditions may not have been attained. Nonetheless, the data indicate that the protein is denatured by exposure to low pH and suggest that denatured apoferritin has more bound protons between pH 3 and 8 than does native apoferritin at equivalent pH.

Titration of apoferritin above pH 9 (Fig. 3), in contrast to titration in other pH regions, was not consistent with different preparations, but the source of this inconsistency remains unclear. As an aid in interpreting the data above pH 9, different preparations were titrated spectrophotometrically and found to give the same tyrosine ionization curve, indicating that differences in tyrosine ionization were not the cause of the scatter in the data. Tyrosine ionization data are shown in the inset of Fig. 3. Tyrosine ionization does not begin until pH 11 as evidenced by changes in absorbance at 245 and 295 nm. The pH at which tyrosine ionization begins is high even when expected (11, 12) electrostatic shifts are allowed for. Above pH 11, the magnitude of the absorbance changes indicates that all 5 tyrosines titrate (complete ionization of a single tyrosine gives changes in ε of 11,000 and 2,500 at 245 and 295 nm, respectively (32)), but the steepness of the titration curve between pH 11 and 12, and the appearance of irreversibility on back-titration from pH 13 indicate that conformational changes accompany tyrosine ionization. Collectively, the data indicate that the 5 tyrosines of apoferritin are abnormal, in agreement with the findings of Crichton and Bryce (29) that only one of the 5 tyrosines can be nitrated and that the pK of the nitrated tyrosine is abnormally high.

An interesting feature of the spectrophotometric titration data is that no changes at 245 nm occur at pH values below those associated with tyrosine ionization; i.e. no changes occur at 245 nm which are not accompanied by the expected (32) 295 nm changes associated with tyrosine titration. Apoferritin has 3 half-cystine residues per subunit (2, 19). The ionization of cysteine-SH groups should be accompanied by changes at 245 nm of sufficient magnitude to be observed under the conditions used here and generally occurs with a pKₐ of 8 to 9; i.e. the ionization of a mercaptan is accompanied by changes in absorbance at 245 nm approximately one-third that of tyrosine at the same wavelength (32). Our data therefore suggest that the half-cystines of horse spleen apoferritin are either largely not in the —SH form or not available to solvent. This is supported by our observation that no more than 0.2 —SH groups per apoferritin subunit are available for reaction with 5,5-dithiobis(2-nitrobenzoic acid), although a single —SH is reactive in both ferritin and apoferritin to bromoacetic acid (Table II). Since, in our hands, the product of reaction with bromoacetic acid was insoluble, the results suggest to us that none of the apoferritin sulfhydryl groups are available in the native protein, but that one becomes available if the conformation is altered. These results are to be compared with those obtained elsewhere (8) which suggested that one apoferritin sulfhydryl per subunit, is available to both 5,5-dithiobis(2-nitrobenzoic acid) and to N-ethylmaleimide.

Further data on side chains potentially contributing to titration above pH 7 were obtained by formal titration; in both ferritin and apoferritin approximately 6.7 lysines titrated at pH 9 at 25° after addition of formaldehyde (HCHO) (Table II). There are 9 lysine residues per apoferritin subunit (2, 19). The results indicate either that 2.3 residues are deprotonated at pH 9 in the absence of HCHO or, more likely, that only 7 of the 9 residues are available for reaction with HCHO. Crichton and Bryce have observed that only 7 of the 9 lysines can be guanidinated (29) and analysis of the titration curve (see below) suggests that no lysines are deprotonated below pH 5.5 and that a maximum of only 0.5 lysine is deprotonated at pH 9, since a total of only 0.5 group titrate between 8.5 and 9.

In order to aid in the interpretation of potentiometric titration data between pH 5 and 8, the enthalpy of ionization was determined from the effect of temperature on titration behavior. Continuous titrations at 8° and 41° were compared with those at 25° after independently establishing the effect of temperature on pH at a single value of h, and ΔH was calculated as a function of h from the relationship (11):

$$\Delta H = 2.3R \left( \frac{\Delta \rho H}{\Delta (1/T)} \right)$$

Results are shown in Fig. 4. Although ΔH values in the neutral pH region were somewhat lower in the 25-41° range than between 8 and 25°, the results indicate a gradual increase in ΔH from values of 1 kcal/mol at pH 4.8 characteristic of carboxyl titration (11) to a value of about 6 kcal/mol near pH 7 (25°) characteristic of imidazole titration (11); above pH 7.8, ΔH increases to values above 10 kcal/mol, signifying that α-NH₂ or ε-NH₂ groups, or both, are the predominant residues above this pH. Assuming normal ΔH values for all groups, the data suggest that the two groups titrating between the pl, (4.96) and pH 5.6 at 25° are almost exclusively carboxyl groups, since the average ΔH in this region is 1.2 kcal/mol. Between pH 5.6 and 7.3 at 25° the four groups that titrate appear to be a mixture of carboxyl and imidazole groups; assuming a ΔH of 6

| Reaction with | Number of Reactive Residues per subunit |
|--------------|------------------------------------------|
| 5,5-dithiobis(2-nitrobenzoic acid) | Apoferritin | Ferritin |
| 0.2—SH | — | — |
| 6.7 Lys | 6.6 ± 0.4 Lys |
| Reactivity with formaldehyde | 48 h | 1 week |
| 1.0—SH⁺ | 0.6 —SH⁺ |
| 2.3 His⁺ | 2.0 His⁺ |
| 1.2—SH⁺ | 1.3 —SH⁺ |
| 3.1 His⁺ | 3.1 His⁺ |

*Not determined.

1. The sample of ferritin used for formal titration was one which had been freshly recrystallized and which therefore showed no pH drift until pH 8.5 and only a slow pH drift at pH 8.9. To minimize error in the formal titration with ferritin, titrations were conducted rapidly; the scatter in the data reflects the consequent slight uncertainty in titration end point.

2. Determined as s-carboxymethylcysteine.

3. Histidine reactivity was determined by subtracting the number of histidine residues found on amino acid analysis of the reacted protein from the original total histidine content, since the carboxymethyl histidine derivatives were not well resolved by the automated analysis system used. As such, the number of reactive histidines represents a maximum value.
Linderstrom-Lang analysis (11, 12) is possible. The analysis present in ion-pair linkage with carboxyl groups (29) but other reasons for their lack of reactivity (cf., lysine acetylation) lysines (see above), Crichton and Bryce report an unreactive gral (2, 19) which may be a reflection of subunit heterogeneity polypeptide chains per subunit (4) with one having a free o-NH, terminus is acetylated (33), but there may be two ritin in which add uncertainty to any attempt at a rigorous and 8. This conclusion is tentatively supported by carboxyme-week of reaction with bromoacetic acid (Table II).

There are several ambiguities about the structure of apofer-ritin which add uncertainty to any attempt at a rigorous analysis of its titration behavior. There is evidence that the α-NH₂ terminus is acetylated (33), but there may be two polypeptide chains per subunit (4) with one having a free α-NH₄⁺; therefore may be one or two α-carboxyl groups per subunit. The number of arginine residues, 9.5, is noninte-gral (2, 19) which may be a reflection of subunit heterogeneity or of analytical uncertainties. In addition to the 2 unreactive lysines (see above), Crichton and Bryce report an unreactive arginine (29); it has been suggested that these residues are present in ion-pair linkage with carboxyl groups (29) but other reasons for their lack of reactivity (cf., lysine acetylation) cannot be precluded. Nonetheless, given the available facts, a self-consistent analysis of the apoferritin data using a modified Linderstrom-Lang analysis (11, 12) is possible. The analysis (Table III) assumes that the 9 nonreactive histidines are masked in the unprotonated form, their protonation in the acid-denatured protein accounting for the tentative difference in h between native and denatured proteins in the pH region 5 to 8. (It can also be shown that the pl of apoferritin cannot be accounted for if the 3 unreactive imidazoles are assumed to be charged at the pl, unless three positive charges are assumed removed from elsewhere in the molecule). Three histidines are assumed to titrate normally, although the normal titration of only 2 histidines would also be compatible with the data, given the small uncertainties in arginine and amide analyses. Two lysines and one arginine are assumed to be buried in the protonated form with 3 side chain carboxylate ions. One α-NH₂ and one α-COOH are assumed per subunit, but the results could accommodate two α-COOHs with little change in fit, particularly if one of the buried histidines is assumed buried in the protonated form. The results could also accommodate the absence of a titratable α-NH₂ if it is assumed that the principal group titrating between pH 7.8 and 8.5 is an abnormal histidine (rather than an α-NH₄⁺ that titrates with an abnormally high ΔH and is unreactive to bromoacetate. The pK and electrostatic interaction factor w characteristic of each titratable class were obtained by successive approximations using the enthalpy of ionization data as a guide to histidine titration and the relationship:

\[ pK' = pK_{\text{int}} - 0.868\text{er}(\bar{Z}) \]

where pK' is the apparent pK at any net charge (\(\bar{Z}\)) and pK_{\text{int}} is the intrinsic pK for that class. (\(\bar{Z}\)) was calculated assuming that binding of ions other than H⁺ was constant over the pH region studied. No attempt was made to optimize the fit of the data, as by computer analysis, because of the tentative nature of some of the assumptions on which the analysis was based. Instead, emphasis was devoted to minimizing the number of adjustable parameters (i.e. the number of classes of titratable groups and the variability of w among different classes).

Titrations of all classes of groups were then summed to give the theoretical curve, shown in Fig. 3; the parameters describing the titration curve are shown in Table III. The curve is in good agreement with the experimental data between pH 3.5 and 8.5 without postulating any significant lysine titration below pH 8.5. Above pH 8.5 the data can be accommodated using titration parameters for the 7 lysines well within the normal range, but the spread of the data does not warrant a rigorous analysis in this pH region. The analysis is internally consistent in that it predicts a net charge on the protein alone of 0 at the observed apoferritin isoeionic pH in KCl. The deviation of the theoretical curve from the data below pH 3.5 is gratifying since it is in exactly this pH region that absorbance and CD data (see above) indicate that conformational changes occur. Agreement between the experimental data, titration theory, and the amino acid composition below pH 3.5 can be obtained by allowing w to gradually decrease below pH 3.5 and allowing buried carboxyls and imidazoles to re-enter H⁺ equilibrium, but we do not consider continuous titration data near pH 2 sufficiently accurate to warrant a detailed analysis. However, it is relevant to note that the titration analysis, in addition to postulating only 3 normal histidines, postulates two classes of titratable side chain carboxyl groups. The intrinsic pK of the principal class (3.90) is slightly lower than would be predicted for a mixture of aspartyl and glutamyl side chains (34), while that of the more basic class (5.56) is decidedly high. While we do not believe that the above analysis of the carboxyl titration is unique in its fit with the data, it can readily be demonstrated that no single values for pK, and w will fit the carboxyl region of the titration curve and that the best fits assume at least two classes of carboxyl groups whose differences in intrinsic pK exceed the known differences (34) between the intrinsic pK values of aspartate and glutamate side chains.

Values of w in Table III are calculated per subunit. Such a calculation ignores the fact that the subunits are aggregated.
give an isoelectric pH of 4.4 in acetate buffer (35). If the value of 4.4
suggesting a change in apoferritin conformation. It is also
between pH 8 and 3.5, with deviations below pH 3.5 again
relevant that the 1
with small changes in pKint; the parameters fit the data well
and show an expected decrease in w at the higher ionic strength
for the data at 0.16 ionic strength, are also included in Table III
derived from analysis of the data, using the same treatment as
shown in the inset in Fig. 5. Preliminary titration parameters
were collected only from pH 5 to 8.3 and from pH 5 to 3 and are
ment of equilibrium or with reversibility. For this reason, data
of comparable size to apoferritin subunits (26).

Precipitate did not appear to interfere with the rapid attain-
dition under these conditions was accompanied
in part for subsequent comparison with ferritin under identical
conditions. Titration under these conditions was accompanied
with the greater stability of ferritin to low pH (see above) or
during the time course of the titration. Back-titration data
suggests a more rapid reversal of acid denaturation in ferritin
than of apoferritin (Fig. 3) is in agreement
rationale for this superposition is presented under "Discussion."
Forward-titration data from pH 4.5 to 11 and from 4.5 to
are shown, as are back-titration for ferritin from pH 7.5, 11,
and 2. Back-titration from pH 2 deviates only slightly from the
forward-titration; the lesser degree of irreversibility on back-
titration of ferritin than of apoferritin (Table II). The effect of pH on the solubility of ferritin was similar to its effect on apoferritin solubility. The titration of ferritin at 20° in 0.16 M KC1 is shown in Fig. 5 and compared with that of apoferritin. The titration curves of the two proteins are superimposed at the isoelectric pH of 4.4 in acetate buffer (35). If the value of 4.4
into a spherical shell, but no simple model can accommodate this
type of aggregation and the method of calculation does not
affect the sensitivity of the analysis to changes in w over the
course of the titration. Interestingly, the calculated value of w
is only slightly higher than that estimated for other molecules
of comparable size to apoferritin subunits (26).

Titration of Apoferritin in 1 M KC1—Titration studies of
apoferitin in 1 M KC1 were carried out, in part to determine
the effect of ionic strength change on titration parameters and in part for subsequent comparison with ferritin under identical
conditions. Titration under these conditions was accompanied
by extensive precipitation on addition of acid, although the precipitate did not appear to interfere with the rapid attainment
of equilibrium or with reversibility. For this reason, data
were collected only from pH 5 to 8.3 and from pH 5 to 3 and are
shown in the inset in Fig. 5. Preliminary titration parameters
derived from analysis of the data, using the same treatment as
for the data at 0 16 ionic strength, are also included in Table III
and show an expected decrease in w at the higher ionic strength
with small changes in pKint; the parameters fit the data well
between pH 8 and 3.5, with deviations below pH 3.5 again
suggesting a change in apoferritin conformation. It is also
relevant that the 1 M KC1 apoferritin titration data, because of
the sharper inflections inherent in the data, confirm that only
eight groups (attributed in our analyses to four carboxyls,
three imidazoles, and one α-NH₂) titrate between the isoionic
pH and the pH (8.3) at which lysine should begin to titrate
(Fig. 5, inset).

Comparison of Ferritin and Apoferritin Behavior—Limited
chemical modification studies of lysine, histidine, and cysteine
were also applicable to KC1 solutions, the effect on calculated values
of pKint at 0.16 ionic strength would be a decrease of approximately
0.05 pH unit.

*The value of w was deliberately set at 0 to simplify curve-fitting.

were also applicable to KCl solutions, the effect on calculated values
of pK_{iso} at 0.16 ionic strength would be a decrease of approximately
0.05 pH unit.

The exact pH at which time-dependent pH drifts were observed
was a function of the state of the ferritin preparation. As preparations
aged, the onset of the drift was shifted to lower pH. Thus, in good
preparations, the pH was stable until 8.5 in 0.16 M KC1, while in old
preparations drift was sometimes evident at pH values as low as 7.5.
Preparations which manifested drift at the lower pH values were not
used for titration studies. However, protein recrystallized from such
aged preparations showed normal behavior (no drift until pH 8.5). The
results suggest an influence of the protein on the properties of the core
or on accessibility to the core.

### Table III

| Residue | No. per subunit | Class       | Ionic strength = 0.16 | Ionic strength = 1 |
|---------|----------------|-------------|-----------------------|-------------------|
|         |                |             | pK_{iso} | w   | pK_{iso} | w   |
| α-COOH  | 1              | Titratable  | 3.0     | 0*  | 3.0     | 0*  |
| β,γ-COOH| 3              | Buried (deprotonated) | 5.56 | 0.08 | 5.56 | 0.05 |
|         | 21             | Titratable  | 3.90    | 0.08 | 4.07    | 0.05 |
| Histidine| 3              | Buried (deprotonated) | 6.33 | 0.092 | 6.41 | 0 |
| α NH₂   | 3              | Titratable  | 7.5     | 0*  | 7.5     | 0*  |
| Lysine  | 2              | Buried (protonated) | 9.6    | 0.08 | Not determined |
| Tyrosine| 7              | Titratable  | >12     |     |         |     |
| Arginine| 5              | Buried (protonated) | >12    |     |         |     |
|         | 1              | Titratable  | >12     |     |         |     |

**Charge balance at pL**

| Negatively charged residues | Neutral residues | Positively charged residues |
|-----------------------------|------------------|---------------------------|
| 1 α-COO⁻                    | 3 Imidazole      | 1 α-NH₂⁺                  |
| 22 β,γ-COO⁻                | 4 β,γ-COOH       | 3 Imidazolium             |
| Total - 23                 | 5 Tyr-OH         | 9 ε-NH₂⁺, ε-NH₂⁺          |

*To determine pK_{iso}, the pH at which the charge on the protein
including that contributed by bound ions (i.e. the isoelectric pH) is
zero must be known (11, 12). We have assumed the isoelectric pH to be
pH 4.55 in both 0.16 M KC1 and 1 M KC1. Free electrophoresis studies
give an isoelectric pH of 4.4 in acetate buffer (35). If the value of 4.4
were also applicable to KCl solutions, the effect on calculated values
of pK_{iso} at 0.16 ionic strength would be a decrease of approximately
0.05 pH unit.

The exact pH at which time-dependent pH drifts were observed
was a function of the state of the ferritin preparation. As preparations
aged, the onset of the drift was shifted to lower pH. Thus, in good
preparations, the pH was stable until 8.5 in 0.16 M KC1, while in old
preparations drift was sometimes evident at pH values as low as 7.5.
Preparations which manifested drift at the lower pH values were not
used for titration studies. However, protein recrystallized from such
aged preparations showed normal behavior (no drift until pH 8.5). The
results suggest an influence of the protein on the properties of the core
or on accessibility to the core.
increasing with increasing pH. That these effects are due to titration of the core is shown by the following study. On allowing a solution of ferritin at 25° to stand in a CO₂-free atmosphere overnight at pH 11, an additional 43 groups per subunit titrated; these were not due to CO₂ absorption, since control solutions under the same conditions did not pick up CO₂. By any calculation, the number of extra groups titrating greatly exceeds the number of potentially titratable groups on the protein at pH 11 and can only be attributed to titration of elements of the core. It is not certain which residues of the core titrate in the pH 8 to 11 region, but preliminary calculations suggest that the number of titratable groups here is at least 4 times greater than the known (1, 2) total phosphate content and may therefore include the titration of water bound to core ferric ions.

Between pH 5.5 and 3, the titration curves of ferritin and apoferritin are virtually identical. Between pH 3 and 2.3, slightly fewer groups titrate in ferritin relative to apoferritin, the difference here undoubtedly being due to the greater stability of ferritin to acid denaturation in this pH region, as evident from CD studies (see above). However, two additional groups appear to titrate in ferritin relative to apoferritin between pH 5.5 and 7.5 and the titration of additional groups in ferritin is also evident near pH 2. As cited above, differences between the two proteins above pH 8 are clearly attributable to the ferritin core and are accompanied by drift during forward-titration of ferritin above pH 8 and irreversibility on back titration. It is therefore of interest that we have observed that titration of the extra two groups in ferritin between pH 5.5 and 7.5 is reproducible, time-independent, and almost completely reversible. Thus, with good ferritin preparations, rapid titra-

†Horse spleen ferritin is heterogeneous with respect to iron content and includes a significant content (20 to 25%) of natural apoferritin (2). It is possible therefore that differences seen here between ferritin and its derivative apoprotein underestimate the real differences between the iron-loaded protein and the iron-free protein.
H+ Ion Interactions of Ferritin and Apoferritin

...ion from pH 5 to 7.5 gives the same number of titratable residues as slow titration, no drifts are noted at pH 7.5 and back-titration (Fig. 5) largely follows the forward-titration curve. In an effort to identify these extra titratable residues, the $\Delta H$ of ionization of titratable residues in ferritin between pH 4.9 and 8.5 was determined as shown above for apoferritin. The results are shown in Fig. 4. In the pH region 5.5 to 7.5, the $\Delta H$ values indicate that the additional groups titrating in ferritin relative to apoferritin must have a $\Delta H$ of ionization of at least +5 kcal/mol, suggesting their identity as histidine side chains to which values of $\Delta H$ of ionization of 6 to 7 kcal/mol are typically (11) assigned. Phosphate groups from the ferritin core are alternative contenders for the role of these additional residues, since the second ionization of phosphate typically occurs near pH 6 to 8; although the $\Delta H$ of this second ionization is approximately +1 kcal/mol in simple molecules (11), there are compelling reasons (see “Discussion”) for believing that phosphates should not be precluded on this basis.

The relationship between the titration curves of ferritin and apoferritin seen at 0.16 ionic strength extends to titration studies in 1 M KCl. In Fig. 5 (inset) the titration curves of the two proteins in 1 M KCl at 25°C are compared, again illustrating the identity in titration behavior between pH 3 and 5.5 and the titration of approximately 2 extra residues in ferritin relative to apoferritin between pH 5.5 and 7.5.

**DISCUSSION**

The isoelectric pH of a protein ($pI_1$) is that at which the intrinsic net charge on the protein (i.e. the charge exclusive of bound ions) is zero. When a neutral salt, such as KCl, is added to an isoionic protein, the protein remains isoionic, even if ions are bound, and the intrinsic net charge on the protein remains essentially zero provided that the protein concentration is high relative to the $H^+$ ion concentration, as is the situation here (11). The isoelectric pH of a protein ($pI_1$) is that at which the total charge on the protein (i.e. the charge including that contributed by bound ions) is zero (11). Any interpretation of the isoionic titration properties of ferritin and apoferritin must reconcile their similar isoionic points in the absence of salt with the divergent effects of salt on the isoionic points of each, as well as with reported identical values of $pI_1$ and electrophoretic mobility of both (see below). As it turns out, these considerations place severe restrictions on the number of possible interpretations of the data. For example, changes in isoionic pH ($\Delta pI_1$) accompanying addition of monovalent ions to proteins are generally interpreted as originating in a change in net protein charge and its accompanying electrostatic effect on $H^+$ ion association constants (11, 31) such that: $\Delta pI_1 = -0.868wZ$, where $Z$ is the charge introduced by the bound ions and $w$ is the same electrostatic interaction factor as that derived from $H^+$ ion titration analysis. This interpretation does not appear to be applicable in its simplest form to the different pH changes accompanying KCl addition to isoionic ferritin and apoferritin since it would lead to the conclusion that there are large differences in total charge between the two proteins at the same pH. Thus, using the value of $w = 0.08$ found for carboxyl titration, data in Fig. 2, would indicate a total charge of approximately +1 per subunit on ferritin at its isoionic pH (4.48) in 0.16 M KCl and a total charge of -6 per apoferritin subunit at its isoionic point in 0.16 M KCl (pH 4.96). Since apoferritin adds only 2 protons per subunit (Fig. 3) on going from pH 4.96 to 4.48, the two proteins would therefore be predicted to differ by a charge of -5 per subunit at pH 4.48 and the isoelectric point of apoferritin would be predicted from the titration data to be 0.9 pH unit lower than that of ferritin in 0.16 M KCl. However, horse spleen ferritin and its derivative apoprotein have been shown to behave identically on free electrophoresis in several different buffers (35) and on isoelectric focusing (36), and therefore probably have similar charges in KCl. Therefore, in our interpretation of the data, a somewhat different basis for the changes in isoionic pH accompanying KCl addition will ultimately be invoked.

A useful starting place in attempting to formulate a model that accounts for the behavior of the system is to consider the virtual identity in the titration behavior of the two proteins between pH 3 and 5.5 at the two ionic strengths studied and their apparent similarity in lysine and histidine availability to modifying reagents. Since titration behavior is sensitive to protein shape and to the number, nature, and environment of available titratable residues, these results suggest that the conformations of ferritin and apoferritin are largely similar and that differences in titration behavior of the two above pH 5.5 and in their ion-binding properties are a reflection of additional titratable residues in the ferritin core, or of a highly localized difference in conformation or environment of amino acid residues at the core-protein interface. The lower $pI_1$ value for ferritin than for apoferritin in KCl can therefore be explained as follows. Per subunit, 2.2 protons must be added to apoferritin at its $pI_1$ in 0.16 M KCl (pH 4.96) to reach the $pI_1$ of ferritin in 0.16 M KCl (pH 4.48), indicating that ferritin (exclusive of bound salt) has approximately two additional negatively charged residues per subunit or two fewer positively charged residues, at pH 4.96, relative to apoferritin. The similar chemical reactivities of the histidines and lysines in the two proteins suggest that the latter alternative is less probable than the former. However, any additional negative charges in ferritin probably do not originate from carboxyl groups since the titration curves of both proteins appear identical in the carboxyl region. The ferritin core contains inorganic phosphate and it is generally believed that a high fraction of the phosphate is near the core surface (2). We postulate that the two additional negative charges on ferritin at pH 4.96 arise from two such surface phosphate residues per subunit, each of which, by analogy with the known ionization behavior of phosphate (11), would be anticipated to carry a unitary net negative charge at pH 4.96. Moreover, since the first phosphate $pK_a$ is generally near 2 in model compounds, this assumption is consistent with the observation that no additional groups in ferritin (relative to apoferritin) protonate until approximately pH 2 (Fig. 3), at which pH the data suggest that more protons are added to ferritin than to apoferritin. Note that the assumption that the extra negative charges on ferritin arise from the core provides the rationale for our superposition of the titration curves of the two proteins at pH 4.96; i.e. differences in the absolute number of protons dissociated from isoionic ferritin (protein plus core) relative to apoferritin (protein) reflect the contribution of the core. The number of protons dissociated from the proteins alone are the same in both cases at pH 4.96.

*In principle, the relationship between $\Delta pI_1$ and the absolute number of bound ions is valid only at constant ionic strength, since changes in ionic strength lead to changes in activity coefficient.
same phosphates of the extra groups that titrate reversibly in ferritin between pH 5.5 and 7.5; i.e. the second ionization of inorganic phosphate occurs with a pKₐ of approximately 7.0. The high ΔH of ionization of the second phosphate ionization could be attributed to interactions between the surface phosphates and the rest of the core. To account for the full spectrum of facts, however, more complex assumptions are necessary. Below, we suggest several models that are tentatively compatible with data.

The simplest model assumes that surface phosphates are both the source of the additional intrinsic negative charges on ferritin in KCl at pH 5 and provide the additional reversibly titratable ferritin residues at pH 5.5 to 7.5. It additionally assumes that anion binding sites, irrespective of their nature, are equally available in both ferritin and apoferritin and that differences between the two proteins arise solely from the presence of cation binding sites on the ferritin core which are absent in apoferritin. To account for the identical values of pI, for both proteins in the absence of salt, it assumes that the surface phosphates are poorly solvated and remote from positively charged groups on either the protein or core. Thus, although phosphates would be expected to carry a unitary negative charge at pH 4.5, these phosphates will not do so in the absence of available counterions. When salt is removed, the effective phosphate pKₐ is raised sufficiently such that the phosphates are electrically neutral at pH 4.58 and do not contribute to the observed pI. The observed values for both proteins in the absence of salt are therefore the same because they are determined only by the protein and not by the core. In the presence of KCl, each phosphate binds K⁺ and liberates a proton, which is then largely added back to the protein carboxyl groups by the usual buffer mechanisms, as shown schematically below.

\[
\text{OH} - \text{P} - \text{OH} \quad \text{COO}^- \quad \text{NH}_3^+ \quad + \quad \text{K}^+ \rightarrow \text{HO} - \text{P} - \text{O}^- \quad \text{K}^+.\]

```
Water, pH 4.58
Net intrinsic charge = 0
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Note that this mechanism keeps the intrinsic charge on the protein (plus core) at pI unchanged by the addition of KCl as necessary (see above); additionally, the core remains electrically neutral when bound ions are considered, accounting for the identical electrophoretic mobilities and pI values of both ferritin and apoferritin. The apparent preference of the core for K⁺ relative to Na⁺ can be explained by invoking residues additional to phosphate at each cation binding site such that stereoechemical factors became operative or, if the hydrated ion is the bound species, invoking a preference for the smaller size of hydrated K⁺ (37) relative to hydrated Na⁺.

The above model has several limitations. First, it does not lead necessarily to the conclusion that the second pK of the phosphate will fall in the correct pH region to account for the extra titratable groups in ferritin in the pH region 5.5 to 7.5, since the phosphate is now part of a K⁺ complex. Second, we have noted that all our ferritin preparations behave identically in the pH region 5.5 to 7.5 irrespective of the horse(s) from which they were derived. This consistency is somewhat easier to reconcile with a model which assigns the extra groups titrating in the pH 5.5 to 7.5 region to no other residues of the protein, or to core elements specifically bound to residues of the protein, rather than to surface phosphates that may only be randomly distributed. At least two alternative but highly speculative models will be cited briefly that account equally well for the data and which address themselves to the difficulties inherent in the first model. Both assume that the extra negative charges on ferritin at pH 5 in KCl originate from phosphate, but other details differ. One such model places the core surface phosphates of ferritin in ion-pair linkage with the same histidines that are "buried" in the unprotonated form in apoferritin. The titration of these histidines is assumed to be the source of the extra titratable ferritin residues in the pH region 5.5 to 7.5, accounting for the high ΔH of titration of these residues. The phosphate hydroxyl that is not in ion-pair linkage is assumed to be the source of protons displaced by K⁺.

This model is not at odds with the equal inaccessibility of 3 ferritin and apoferritin histidines to carboxymethylation, since the ferritin histidines remain inaccessible, even if protonated, by virtue of their pairing with phosphate. A second model, in contradistinction to the above two, assumes that differences in K⁺ binding by ferritin and apoferritin reflect not only K⁺ binding by the ferritin core, but also the presence of anion binding sites on apoferritin that appear absent in ferritin because they are occupied by core phosphate residues; the extra titratable residues in ferritin in the pH region 5.5 to 7.5 are assumed to be phosphates. This model can be shown to necessitate that the anion binding sites in apoferritin lose a proton when KCl is removed at pH 5, but this difficulty is not judged to be insurmountable. Finally, we should note the possibility that the extra groups on ferritin which titrate might arise from the ionization of water molecules coordinated to iron; such iron could represent molecules at the core surface or face of the protein shell and occupied by phosphate in ferritin, would have a tendency to deprotonate in the absence of salt.

The scheme shown also demonstrates again why one additional carboxyl per phosphate is protonated at the pI of ferritin relative to that of apoferritin in KCl and why, accordingly, the pI of ferritin occurs at lower pH. At any given pH, the charges on the protein components of both ferritin and apoferritin are the same.
arise from iron bound to sites on the protein in equilibrium with the core. The latter type of site has been suggested elsewhere (38), although it should be stressed that the titration data here show little evidence of their existence. For example, the identical carboxyl titration in both ferritin and apoferritin, the equal lysine availability in both proteins, and the nonavailability of cysteine and tyrosine in apoferritin would leave only histidine residues as likely sites for strong iron coordination.

The above models assume that the principal differences in the titration behavior and stability of ferritin and apoferritin are not the result of differences in conformation; i.e. no differences are found between the titration behavior of the two proteins which cannot be attributed solely to their differences in core content. Even differences in stability between the two proteins as observed here and elsewhere (9, 10) can be reconciled with the same conformation for both if stabilizing interactions between the core and protein, such as the salt-bridges we have invoked, are present in ferritin. The question therefore still remains as to whether configurational differences between ferritin and chemically prepared apoferritin, suggested by differences in the far-ultraviolet CD spectra, are present. Results here suggest either that any configurational differences between the two proteins are localized within regions of the molecules which contain few, if any, titratable residues, or that CD differences between the two are artifactual. Such an artifact could arise if the core affects the CD of ferritin chromophores, either because the high absorbance of the core leads to an absorption flattening (39), or because the core provides a different refractive index (40) for those residues with which it is in direct contact, or because of induced optical activity in the surface elements of the core which may be in contact with the protein. Preliminary measurements suggest that any CD flattening by the core should be relatively small and that no measurable optical activity is induced in transition of the core that occur in the visible wavelength region. However, these effects as well as possible refractive index contributions of the core deserve further exploration.

In conclusion, our studies support a marked similarity in the properties of ferritin and apoferritin. However, surprising differences in response of the two proteins to KCl have been demonstrated and would seem to be a profitable area for further investigation. For example, specific ion-binding studies are needed to determine whether these differences are due solely to cation binding by the core or whether, as well, anion binding sites in apoferritin have been occupied in ferritin by the core. Additionally, we have placed major emphasis on ferritin phosphates in the interpretation of our data, both in their potential role as proton and K’ binding sites, and their possible interactions with residues of the protein. The role of phosphate in ferritin is uncertain, particularly since iron can be incorporated into apoferritin in vitro in the absence of phosphate (2). It is of interest to point out, however, that the known greater stability of ferritin than of apoferritin necessitates that there be some interaction between the protein and the core; such an interaction could well involve phosphates. Additionally, it is possible that the cation-binding properties of the ferritin core, which we ascribe to phosphates, play a role in the crystal growth stage (41) of the iron incorporation process. Comparative physical studies of natural ferritin and ferritin reconstituted in the absence of phosphates would therefore be of interest.

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