The Phosphate on the Iron Core of Horse Spleen Ferritin as Studied by a ^1H NMR Probe Method

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Glycylglycylglycylglycine and tetramethylammonium chloride have been used as ^1H NMR probes to study the state and role of the phosphate on the surface of the iron core of ferritin. ^1H NMR signals of the probe molecules in a ferritin solution do not show any observable paramagnetic shift, and the linewidths are very small compared to an aqueous iron(III) solution which contains the same amount of iron. Iron of ferritin is markedly different from that of aqueous iron(III), but quite resembles that of synthetic iron polymer. It has been shown that the parameter k, which is the linewidth for the NMR probe divided by the concentration of iron, is dependent on the content of the phosphorus in ferritin. The k value for glycylglycylglycylglycylglycine has a maximum in the physiological pH range 6—7, whereas that for tertramethylammonium chloride in which the charge does not change in the whole pH range gives a sigmoidal curve. From the pH dependence of the k values for tetramethylammonium chloride, the pK_a value of the phosphate group on the surface of the iron core has been estimated at 7.0 ± 0.2.

Ferritin, an iron storage protein distributed widely in both plant and animal kingdoms, consists of a shell of protein subunits surrounding the iron core which contains up to approximately 4000 iron atoms.[1,2] Several methods such as X-ray diffraction,[3] electron microscopy,[4,5] and neutron scattering[6] have so far been applied for the investigation of the structure of the iron core. The magnetic properties of the iron core have also been studied by the measurements of magnetic susceptibility[7,8] and Mössbauer effect.[9] It is well established that about 1 to 1.5% phosphate is contained in the iron core, and it has been suggested that the concentration of the phosphate group is higher on the surface than in the inner part of the iron core.[3,10] It has also been suggested that the negatively charged phosphate group is acting as a *go-between* for the binding of the iron core to the protein shell.[1]

Silk and Breslow[11] have carried out the potentiometric titration of apoferritin and ferritin, and observed that ferritin is more stable than apoferritin in the pH range 2.3—3.0, and above pH 8, there exists a large difference in the titration behavior between the two proteins. They have also shown that, compared to apoferritin, ferritin has two more groups per protein subunit which dissociate reversibly in the pH range 5.5—7.5, and concluded that these additional groups are the phosphate group on the iron core. In the present work, high resolution nuclear magnetic resonance (NMR) is used to investigate the structure and magnetic properties of the iron core of ferritin with emphasis on the phosphate group. Because of the large size of the molecule (molecular weight, 445000) and the existence of a large amount of paramagnetic iron, any NMR signal from the protein shell of the native ferritin cannot be detected; only ^1H signal observed is of the solvent water. ^{31}P NMR signal of the phosphate group was also not observable.

In a method presented in this paper, tetramethylammonium ion, glycylglycylglycylglycylglycine, and water are used as the NMR probe to obtain information about the surface of ferritin. When the probe molecules are in close contact with the iron core of ferritin, the NMR signals of the probes are broadened as a result of the magnetic dipolar interactions. The dipolar interactions are strongly influenced by the electrostatic interactions between the probe molecules and the surface of ferritin. In view of this, we use tetramethylammonium ion with a positive charge throughout the pH range examined, and glycylglycylglycylglycylglycine which has at the physiological pH a negative and positive charge at the N and C terminals of the peptide chain, respectively. The water molecule is also used as a neutral probe. On the basis of these experiments, the dissociation constant of the phosphate group on the surface of the iron core will be estimated.

Experimental

Materials. Horse spleen ferritin (crystallized twice, Cd free) was purchased from Nutritional Biochemicals Corporation (lot No. 4871 and 4493), and dialyzed against distilled water before use. Tetramethylammonium chloride, glycylglycylglycylglycine and other reagents used are of reagent grade. Polymeric iron hydrolysate (hereafter designated as iron polymer) was prepared by adding 0.5 M $NaHCO_3$ and 0.05 M NaOH to a 0.03 M ferric citrate solution; the iron polymer thus produced was isolated on a column of Bio-Gel P-30.[12] Iron polymer was also prepared by heating a 0.02 M solution of ferric nitrate at 80—85 °C for 10—20 h, followed by dialysis of the resultant solution.[13]

Analysis. The concentration of iron in ferritin preparations were determined by atomic absorption. No attempt was made to decompose the iron core before the samples were subject to atomic absorption. The iron content determined in this way is in excellent agreement with that obtained by atomic absorption with the iron core decomposed by reducing it with sodium sulfite. The result is also consistent with the value determined colorimetrically by using 2,2′-bipyridyl.[14] Protein concentrations were determined by the Lowry method,[15] and the concentration of the phosphate by a colorimetric method using ammonium molybdate and ascorbic acid.[16]

Instruments. ^1H NMR spectra were recorded using a JEOL PS-100 spectrometer operating at 100 MHz.

Methods. NMR spectra of glycylglycylglycylglycylglycine (Gly_4) were measured in D_2O; in H_2O, due to the NH protons of the amino groups, the signals from the methylene protons of Gly_4 become complicated. The spectrum of tetramethylammonium chloride (TMA) was observed in H_2O. The concentrations of Gly_4 and TMA were constant throughout the experiments (10 mM). The concentration of ferritin in sample solutions was adjusted in either of the following two ways:

1) A small amount of a stock solution of ferritin at high concentrations (approximately 0.2—0.4 M as iron) was added to a Gly_4 or TMA solution. 2) Ferritin solutions were dialyzed against a glycine–HCl buffer solution which contains 10 mM TMA, and iron concentration of the solution was determined after dialysis. In all experiments described in the present work, the pH was changed by dialyzing a solution for 24 h against a 10 mM glycine–HCl buffer, and the dialyzed ferritin solution was added to a 10 mM Gly_4 or TMA solution where the pH was pre-adjusted to that of the ferritin solution added; before and after this treatment, very little change in pH is observed.

Results

^1H NMR Signals of the Solvent H_2O Containing Ferritin and Synthetic Iron Polymer. The H_2O proton signals of aqueous iron(III) solution, ferritin, and synthetic iron polymer solution are reproduced in Fig. 1. ^1H NMR signals of H_2O containing varying amounts of ferritin have the following common features. First, the signal is shifted to high field from that of pure H_2O. Presumably, this is because the bulk susceptibility of ferritin solutions is increased by the presence of a large quantity of paramagnetic iron. The observed difference in chemical shift between the ferritin solution and water would give the bulk magnetic susceptibility of the ferritin iron in water.[17] The result obtained was 3.8 μ_B. This value can be compared to that of Michaelis, 3.7—3.9 μ_B, which was obtained by using powder samples.[7] Second, the H_2O signal in ferritin solutions does not show any paramagnetic shift in the concentration range 0—0.4 M (as iron). This observation is in a marked contrast with a large paramagnetic shift which is observed in an aqueous iron(III) ion solution. Third, the linewidth of the H_2O signal of ferritin solutions is only about 2.5% of that for an aqueous iron(III) solution ($Fe(ClO_4)_3$/2 M $HClO_4$; solution 2, Fig. 1) which has the same iron concentration. Table 1 shows the paramagnetic shifts and linewidths of the H_2O signals of 0.05 M iron(III) and iron(II) solutions. The water signal of aqueous iron(III) solutions (d^5, high spin) always has very large linewidths (200—400 Hz); even a iron(III)–EDTA complex gives a linewidth of the water signal of larger than 100 Hz. It should also be noted that paramagnetic shifts are very small, if any, in the case of ferritin and synthetic iron polymers, whereas they are in the range of 100—200 Hz in the case of aqueous iron(III) and iron(II) solutions.

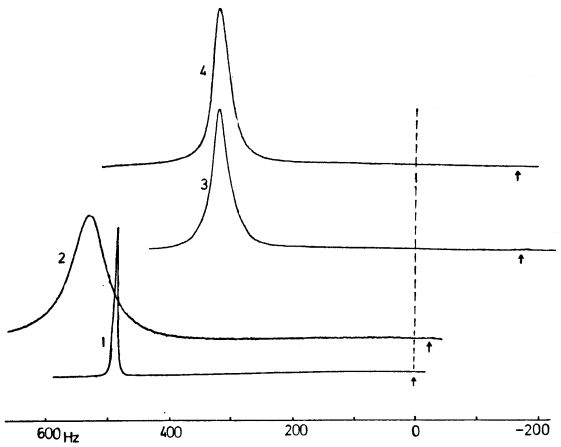

Fig. 1. ^1H NMR spectra of H_2O of 1: pure water, and aqueous solutions containing, 2: ferric ion (0.010 M $Fe(ClO_4)_3$/2 M $HClO_4$), 3: ferritin (0.11 M as iron, pH 7.4), and 4: iron polymer (0.13 M as iron pH 5.5). Arrows indicate the position of the peak of DSS used as internal reference. Locking on external H_2O was used in order to observe a relative shift of the DSS peak in spectra 2, 3, and 4 with respect to that for pure water. The observed shift presumably reflects a change in bulk magnetic susceptibility of the aqueous solutions.

Table 1. The paramagnetic shifts and the linewidths of H_2O solutions containing aqueous iron(III) and iron(II) ions, synthetic iron polymers and ferritin[a]

	Paramagetic shift (Hz)[b]	Linewidth (Hz)[b]
$Fe(ClO_4)_3$/2 M $HClO_4$	224	405
$FeCl_3$/2 M HCl	172	192
$Fe(NO_3)_3$/2 M HNO_3	194	385
$FeSO_4(NH_4)_2SO_4$/2M H_2SO_4	143	12
Fe(III)-EDTA	52	114
Ferritin	≈0	13
Iron polymer	≈0	11

a) The concentration of iron is 0.05 M in all cases.
b) Accurate to ±2 Hz.

As shown in Table 1, the iron polymer markedly resembles the iron core of ferritin; the H_2O signal of solutions of the iron polymer exhibits no paramagnetic shift and the linewidth is quite small. From the chemical shift of the H_2O signal, the magnetic susceptibility of the iron polymer of iron(III) citrate was estimated at 3.5 μ_B.

^1H NMR Spectra of Gly_4 and TMA in the Presence of Ferritin, Synthetic Iron Polymer, and Aqueous Iron(III) Ion. In order to compare more closely the different types of iron mentioned above, a small peptide Gly_4 was used as an NMR probe. Four peaks which correspond to the four kinds of the methylene protons (which from the N terminal will be designated as N, N2, N3, and C) can be observed separately in D_2O. The spectra of Gly_4 in the presence of either one of the three kinds of iron, *i.e.*, aqueous iron(III) ion ($Fe(ClO_4)_3$), ferritin, and synthetic iron polymer, are shown in Fig. 2. Upon adding an aqueous iron(III) solution, differential broadening of the four methylene peaks are clearly observed; the peak from the C terminal first becomes broad. Each of the four methylene peaks shows paramagnetic shift as shown in Fig. 2, A. In contrast to this, when ferritin is added, all four peaks become broader almost uniformly, without any paramagnetic shift. The dependence of the chemical shifts of the Gly_4 signals is plotted in Fig. 3 as a function of the iron concentration.

In the case of the ferritin solutions, although all lines are almost uniformly broadened, the peak from the N terminal broadens a little faster than the others

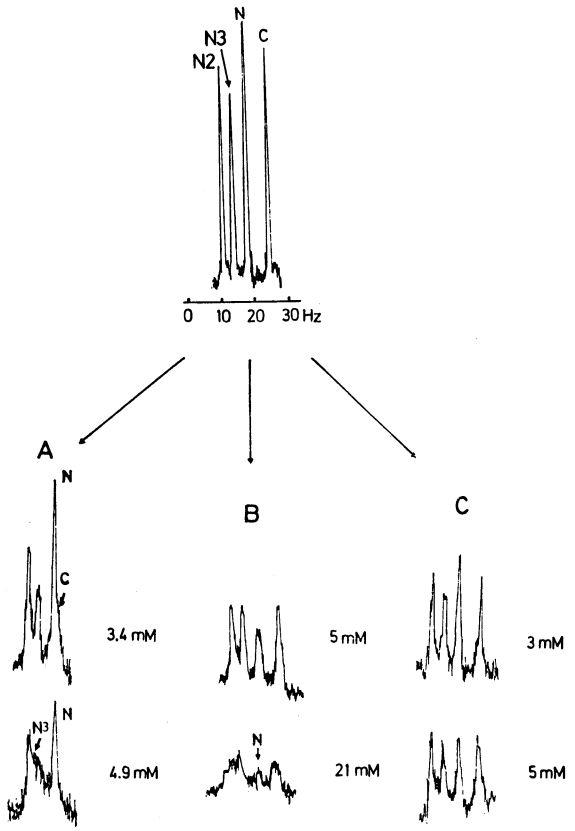

Fig. 2. ^1H NMR spectra of Gly_4 in D_2O in the presence of either one of the three kinds of iron. A, aqueous iron(III) ion ($Fe(ClO_4)_3$), pH 6.8; B, ferritin, pH 6.8; C, synthetic iron polymer, pH 6.8. The concentrations of iron added, and the assignment of the peaks are shown in the figure.

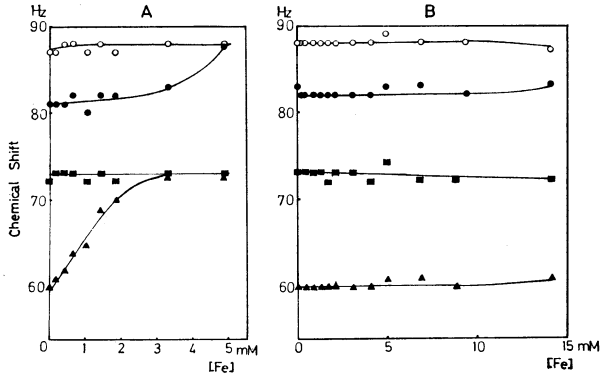

Fig. 3. The dependence of the chemical shifts of the Gly_4 signals as a function of the concentration of iron. (A) aqueous iron(III) ion, (B) ferritin. ▲, C terminal peak; ■, N terminal peak; ●, N3 peak; ○, N2 peak.

(Fig. 4, B). It has been confirmed that the linewidth of the NMR probes examined is not influenced by the presence of apoferritin. This means that the line broadening of the NMR probes is independent of the protein part, and therefore a contribution from the iron core can be observed separately by NMR. In view of this result, the paramagnetic contribution for each peak may be represented quantitatively by using the slope which is obtained from the relation between the

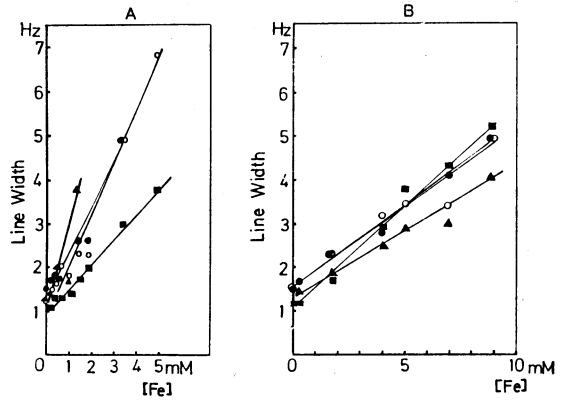

Fig. 4. The linewidths of the Gly_4 signals as a function of the concentration of iron. (A) aqueous iron(III) ion, (B) ferritin. ▲, C terminal peak; ■, N terminal peak; ●, N3 peak; ○, N2 peak.

iron added and the linewidths of the peaks. The observed linewidth ΔH_{obsd} may be written as follows:

$$\Delta H_{obsd} = \Delta H_0 + \Delta H_p, \quad (1)$$

where ΔH_0 is the intrinsic linewidth of each peak and ΔH_p represents the paramagnetic contribution. In the case of fast exchange where exchange between molecules which are under the influence of the iron core, and those which are free in solution is fast enough, the paramagnetic term (ΔH_p) should increase linearly with the iron concentration added. In this case, $k=\Delta H_p/C$ which represents a normalized paramagnetic contribution should be constant, and therefore Eq. 1 may be written as

$$\Delta H_{obsd} = \Delta H_0 + kC. \quad (2)$$

In Fig. 4 the observed linewidths are plotted as a function of the concentrations of iron added. The slopes k obtained from Fig. 4 may be considered as representing the binding ability of Gly_4 to ferritin, and therefore may be used to estimate the binding constant of Gly_4 with iron. The k values obtained are summarized in Table 2. In view of the fact that the k value for the C terminal protons is largest in an aqueous iron(III) solution and that the peak from the C terminal exhibits a largest paramagnetic shift, it is obvious that an aqueous iron(III) ion forms a complex with the carboxyl group of Gly_4. In the case of ferritin solutions, the slopes for the four peaks of Gly_4 are all small, and almost equal to each other. The slope for the N terminal protons are slightly larger than that of the C terminal

Table 2. The observed k values for Gly_4 in H_2O solutions of aqueous iron(III) ion and two different ferritin preparations which contain a different amount of the phosphate

	N2	N3	N	C
$Fe(ClO_4)_3$	1.2 Hz/mM	1.3	0.63	2.0
Ferritin[a)]	0.39	0.53	0.45	0.30
Ferritin[b)]	0.06	0.06	0.11	0.08

a) P/Fe=0.10. b) P/Fe=0.08.

protons. This result may be interpreted in terms of the existence of the negative charge of the phosphate group on the surface of the iron core.

For the sake of comparison, a synthetic iron polymer which does not contain any phosphate group, and also a different batch of ferritin which contains a less amount of the phosphate were examined. It has been confirmed that in the case of the iron polymer, the N-terminal peak is less broad than others as shown in Fig. 2, C. It is shown in Table 2 that the k values for the Gly_4 peaks in the presence of ferritin containing a less amount of phosphate are much smaller, and that the difference in k between the N and C terminal peaks becomes small. Furthermore, it was confirmed that the linewidth of the solvent H_2O signal is independent of the concentration of phosphate in the iron core. Therefore, it may be concluded that the broadening of the Gly_4 peaks is due to the existence of the negative charge of the phosphate on the surface of the iron core. The titration curve of k for Gly_4 shown in Fig. 5 indicates that the curve has a maximum and decrease above pH 7. Most probably, this is because the amino group of Gly_4 loses its charge at high pH and as a result of this, the interaction between Gly_4 and ferritin

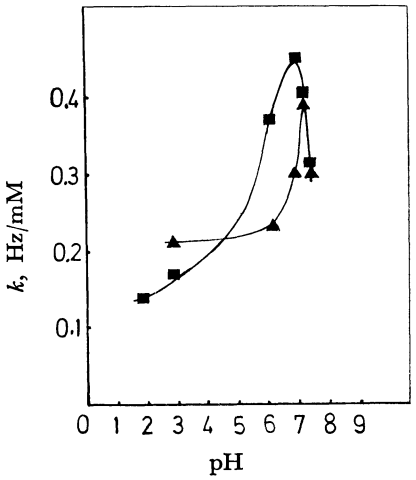

Fig. 5. The titration curve of the k values for Gly_4. ▲, C terminal peak; ■, N terminal peak.

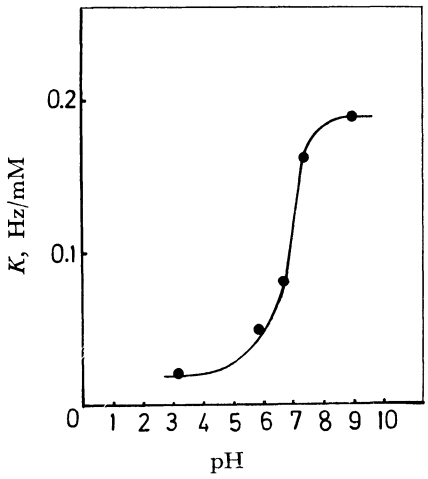

Fig. 6. The titration curve of the k values for tetramethylammonium ion.

becomes weaker in this pH range. TMA in which the charge does not change in the whole pH range was used at the same time as a reference, and the results are given in Fig. 6.

The lienwidths of the peaks for Gly_4 are designated as ΔH_{mono} and ΔH_{di} when the phosphate group exists as mono- and di-anion, respectively. The fraction of the dianion is denoted by x. Then, the observed linewidth in the presence of ferritin may be given by

$$\Delta H_{obsd} = \Delta H_{mono}\cdot(1-x) + \Delta H_{di}\cdot x. \quad (3)$$

This equation is normalized by dividing it by C.

$$k_{obsd} = k_{mono}\cdot(1-x) + k_{di}\cdot x$$
$$= k_{mono} + (k_{di}-k_{mono})\cdot x, \quad (4)$$

where C is the concentration of ferritin (as iron), $k_{obsd}=\Delta H_{obsd}/C$, $k_{mono}=\Delta H_{mono}/C$, $k_{di}=\Delta H_{di}/C$. A plot of k_{obsd} as a function of pH should represent the dissociation of the phosphate, and therefore give the pK_a of the phosphate group. From the plot in Fig. 6 obtained for TMA, a pK_a of 7.0 ± 0.2 for the phosphate is obtained. It is quite interesting that the value obtained is close to $pK_a=7.2$ which has been reported for the second dissociation of an inorganic phosphate ion. In the case of Gly_4, the pH dependence of k is shown in Fig. 5. The curve for Gly_4 has a maximum in the pH range 6—7, suggesting that the interaction between Gly_4 and the phosphate becomes strongest in this pH range. In this narrow pH range, the slope for the C terminal methylene peak is much smaller than that for the N terminal. This suggests that in this pH range the positive charge of the amino group of Gly_4 is attracted to the negative charge of the phosphate group on the iron core.

Discussion

As mentioned previously, the linewidth of the H_2O signal in ferritin solution is quite small, and only about 2.5% of that for an aqueous iron(III) solution which contains the same amount of iron. The H_2O signal of ferritin solutions markedly resembles that for a solution containing synthetic iron polymer. This result suggests that the number of iron(III) ions which are in contact with water becomes very small when the ions polymerize, forming a large iron core. Therefore, a change in the surface area should greatly influence the linewidth. In the case of the ferritin preparation (lot No. 4871) used in most of the present experiment, the ratio of iron to protein is 0.20. The iron core is assumed to be spherical in shape; then in a close packing model[3], the radius of the sphere would be 24 Å and the core could contain 1600 iron atoms. This means that the average surface area per iorn atom of the iron core is about 1/16 of that for a free iron(III) ion. In addition to this, there is a difference in magnetic susceptibility between an aqueous iron(III) ion (5.9 μ_B) and ferritin iron (3.8 μ_B). Therefore, if the linewidth solely due to dipolar interactions between H_2O and the electron spin of iron, a factor of $(3.8/5.9)^2=0.41$ should also be considered for the linewidth of the NMR probes.[18] In the above simple model, a total reduction in linewidth becomes 2.4% ($1/16\times0.41\times100$) which

agrees well with the observed value. A quite similar result has been obtained in the case of a different batch of ferritin preparation (lot No. 4493). Although the quantitative agreement may be fortuitous, it appears that the observed linewidths may be accounted for by the above simple model.

It has been confirmed that the linewidth of the NMR probes examined is not influenced by the presence of apoferritin. This means that the line broadening of the NMR probes is independent of the protein part, and therefore a contribution from the iron core can be observed separately by NMR. In this respect, the present experiment using the NMR probes is essentially different from that by Silk and Breslow who using potentiometry attempted to acquire information concerning the phosphate on the iron core.[11] Since all dissociable groups are observed together in potentiometry, it is generally difficult to distinguish the dissociation in protein from that in the iron core. In fact, they assigned a group which dissociates in the pH range 5.5—7.5 to either histidine in the protein part, or the phosphate group on the iron core. Since the results obtained in potentiometry are based on the difference of the titration curves between ferritin and apoferritin, it is necessary to assume that in the whole pH range examined a difference in conformation between the two proteins, if any, does not influence the titration behavior. However, there is evidence on the basis of circular dichroism spectra that there is actually a difference in conformation in the two proteins.[19] These ambiguities do not occur in using the NMR probe method where information concerning the iron core can be obtained separately.

Evidences which support the role of the phosphate on the broadening of the NMR probes may be summarized as follows: 1) Broadening of the N terminal peak of Gly_4 occurs faster than that of the C terminal peak, indicating broadening of the Gly_4 signals is influenced by the negative charge. It should also be noted that the difference in broadening between the C and N terminal peaks becomes particularly larger in the pH range where the phosphate group dissociates. 2) In the case of the iron polymer which does not contain any phosphate group, the N terminal peak becomes equally broad as the C terminal peak. 3) In a ferritin preparation which contains less phosphate, the broadening of the Gly_4 peaks is less extensive. 4) The linewidth of the H_2O peaks remains constant in the pH range 2—10. From these results it is clear that the broadening of the Gly_4 peaks is primarily due to the phosphate group on the iron core. The protein part of ferritin also has the negative charge. However, the positive and negative charges are distributed over the surface of the protein, and effective negative net charge is -2——-6 in the pH range 5.5—7.5.[11] In contrast to this, the number of phosphate groups in one ferritin molecule is about 290 for the ferritin preparation (lot No. 4493) where the ratio iron/protein is 0.20 and phosphorus/iron is 0.10. If four fifth of these phosphate groups is assumed to exist on the surface of the core,[3] the number of charge on the core is more than two hundred, and becomes twice when the phosphate groups dissociate above pH 7. This result along with more extensive study using a variety of NMR probes now in progress in this laboratory further confirms that the NMR probe method presented here is quite useful in investigating in detail the surface of the iron core.

It should also be pointed out that when the pH of ferritin solutions is adjusted by adding acid or base directly to the solutions as performed in Silk and Breslow's experiments, it is quite likely that the quaternary structure of ferritin is perturbed, particularly in the high and low pH range.

It should be noted that Gly_4 interacts with ferritin most strongly at pH 6—7, and the interaction becomes weak with an increase or decrease in pH (see Fig. 5). In view of this result, it appears that the phosphate on the surface of the iron core interacts with the amino groups on the inner surface of the protein shell, playing a role to bind the iron core to the protein. It is quite interesting that the interaction between Gly_4 and ferritin becomes maximum at pH 6—7, the physiological pH which would be favorable for the binding of the iron core to the inner surface of the protein.

References

1) P. M. Harrison, R. J. Hoare, T. G. Hoy, and I. G. Macara, "Iron in Biochemistry and Medicine," ed by A. Jacobs and M. Worwood, Acadimic Press, London (1974), p. 73.
2) R. R. Crichton, *Struct. Bonding (Berlin)*, **17**, 67 (1973).
3) P. M. Harrison, F. A. Fishbach, T. G. Hoy, and G. H. Haggis, *Nature*, **216**, 1188 (1967).
4) G. H. Haggis, *J. Mol. Biol.*, **14**, 598 (1965).
5) W. H. Massover and J. M. Cowlew, *Proc. Nat. Acad. Sci. U.S.A.*, **70**, 3847 (1973).
6) H. B. Stuhrmann, J. Haas, K. Ibel, M. H. J. Koch, and R. R. Crichton, *J. Mol. Biol.*, **100**, 399 (1976).
7) L. Michaelis, C. D. Coryell, and S. Granick, *J. Biol. Chem.*, **148**, 463 (1943).
8) A. Blaise, J. Freon, J. L. Girardet, and J. J. Lawrence, *C. R. Acad. Sci.*, **265**, 1077 (1967).
9) G. W. Brady, C. R. Kurkjian, E. F. X. Lyden, M. B. Robin, P. Saltman, T. G. Spiro, and A. Terzis, *Biochemistry*, **7**, 2185 (1968).
10) S. Granick, *J. Biol. Chem.*, **146**, 451 (1942).
11) S. T. Silk and E. Breslow, *J. Biol. Chem.*, **251**, 6963 (1976).
12) T. G. Spiro, L. Pape and P. Saltman, *J. Am. Chem. Soc.*, **89**, 5555 (1967).
13) K. M. Towe and W. F. Bradley, *J. Colloid. Interface Sci.*, **24**, 384 (1967).
14) J. W. Drysdale and H. N. Munro, *Biochem. J.*, **95**, 851 (1965).
15) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall *J. Biol. Chem.*, **193**, 265 (1951).
16) P. S. Chen, Jr. T. Y. Toribara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).
17) D. F. Evans, *J. Chem. Soc.*, **1959**, 2003.
18) A. Abragam, "The Principles of Nuclear Magnetism," Oxford, The Clarendon Press, p. 97.
19) G. C. Wood and R. R. Crichton, *Biochim. Biophys. Acta*, **229**, 83 (1971).