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^{1}H NMR Study of Interaction of Probe Molecules with the Phosphate Ion on the Iron Core of Horse Spleen Ferritin

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The surface of the iron core of ferritin was investigated by ^{1}H NMR using small probe molecules which have different sizes and charges. It was shown that the parameter k, which is the linewidth for each NMR probe normalized by the total concentration of iron in ferritin, is dependent on the content of phosphorus in ferritin. At pH 7.4, the k values are 1—10 for positively charged probes, 2×10^{-2}—8×10^{-2} for negatively charged probes and 0.1—0.2 for neutral probes. Above pH 7, the k values for positively charged probes increase with an increase in pH, whereas those for negatively charged probes decrease. By contrast, the k values for neutral probes remain constant over the entire pH range. It was concluded that the k values reflect the mode of interactions of small molecules with the phosphate which exists on the surface on the iron core of ferritin, and that these interactions are determined primarily by the electrostatic force between the small molecules and the negative charge of the phosphate of ferritin. Furthermore, it was shown by using t-butylamine as a probe that K^+ ion binds strongly to ferritin and that chelating agents interact with ferritin in a wide concentration range.

Ferritin is an iron storage protein consisting of a shell of protein subunits surrounding the iron core which contains up to approximately 4000 iron atoms.[1,2] It has been shown that the iron core contains about 1 to 1.5% phosphate[3] and the concentration is much higher on the surface than in the inner part of the iron core.[4,5] It has also been reported that the ratio of phosphate to iron decreases with an increase in the iron content of ferritin.[5] Treffry and Harrison showed that ferritin, which had been reconstituted from iron(II) ion and apoferritin followed by incubation of the product in the presence of phosphate, resembles native ferritin with respect to the concentrations of phosphate incorporated per molecule or per Fe atom, and suggested that in the native ferritin most of the phosphate is adsorbed on the surface of the ferritin iron core after it is formed in the absence of phosphate.[6] May and Fish reported that the diffusion of acetic acid into apoferritin is much slower than that of methanol, glucose, and methylammonium ion.[7] Jones *et al.* showed that iron release from ferritin by polyanionic dihydroflavins is much slower than that by other dihydroflavins.[8]

In a previous paper, we have reported an NMR probe method where small molecules such as solvent water, tetramethylammonium ion and glycylglycylglycylglycine were used to study magnetic properties of the iron core of ferritin and to determine the pK_a values of the phosphate group on the surface of the iron core.[10] Because of the large size of the protein (molecular weight of the protein part, 445000),[1,2] any NMR signal from the protein shell of the native ferritin cannot be detected. It has been confirmed that the linewidth of the NMR probe examined is not influenced even if a high concentration of apoferritin (10 mg/ml) is present, indicating that the probe molecules are little influenced, if any, by the protein part of ferritin. It is unlikely that the paramagnetism of the ferritin iron can contribute to the line broadening of the probe molecules which exist outside the ferritin molecule; the effect of the iron core would decrease with the inverse of the sixth power of the distance.[9] Therefore only a direct interaction between ferritin iron core and the probe molecules, which contact with the iron core inside the protein shell, can be observed by the NMR method.

In the present experiment, the charge and size of the probe molecules were changed systematically. From these results, the state of the phosphate in the iron core will be discussed in detail. The interaction between ferritin and chelating agents, which are known to play an improtant role in the release of iron from ferritin, was also studied by the NMR probe method; the concentration range in which the chelating agents strongly interact with ferritin will be determined.

Experimental

Horse spleen ferritin was purchased from Nutritional Biochemicals Corporation (crystallized twice, Cd free, lot No. 4871, 4493). All chemicals used were of reagent grade. The concentration of iron in ferritin solutions were determined by atomic absorption.[10] Protein concentrations were measured by the Lowry method[11] and phosphorus concentrations were measured by a colorimetric method using ammonium molybdate and ascorbic acid.[12] The pH and concentration of ferritin in sample solutions were adjusted as follows: A stock solution of ferritin was prepared at concentrations of 0.2—0.4 M (as iron), and the pH of the solution was adjusted by dialyzing it against a buffer solution at a desired pH. The buffer solutions used were 10 mM glycine–HCl below pH 3.5; 0.1 M acetate in the pH range 3.5—6.2; 0.1 M Tris–HCl above pH 6.8. The stock solution thus prepared was added to a 10 mM probe solution just before each measurement. Before and after this treatment, very little pH change was observed; precipitation did not occur either. The pH of ferritin solutions was varied from 2 to 10. ^{1}H NMR spectra were recorded using a JEOL PS-100 spectrometer operating at 100 MHz in the conventional cw mode.

Results

Figure 1A shows how the linewidths of the methyl protons of methylamine and acetic acid are influenced differently with increasing concentrations of ferritin. At pH 7.4, the linewidth for methylamine is broadened 4—5 times at a concentration of 0.36 mM (as iron), whereas that for acetic acid remained almost unchanged even in the presence of 3.6 mM ferritin. Figure 1B

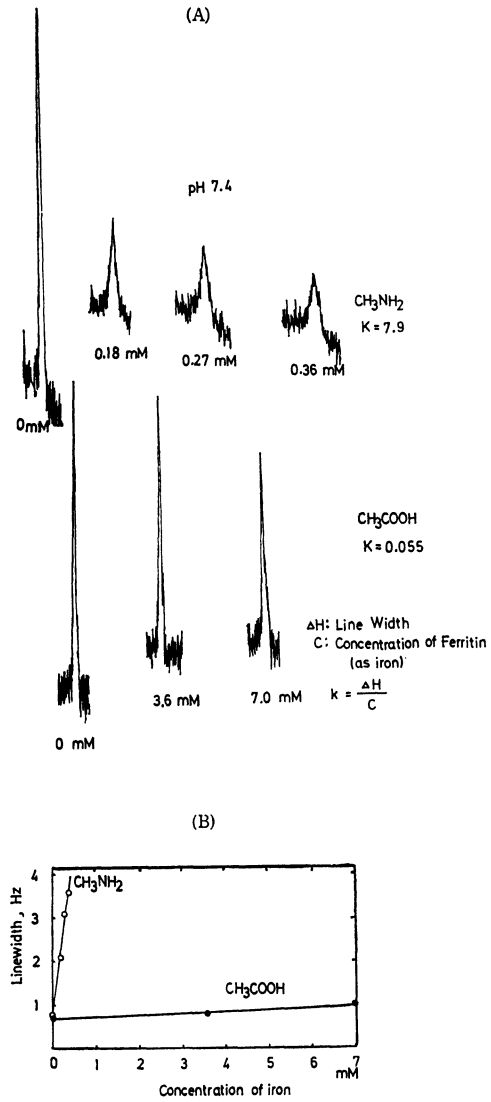

Fig. 1. (A) ^1H NMR spectra of methylamine (10 mM) and acetic acid (10 mM) at pH 7.4 in the presence of ferritin. The concentration (as iron) of ferritin added is shown in the figure. (B) The linewidths of the methyl peaks of methylamine and acetic acid as a function of the concentration of ferritin added.
○: Methylamine, ●: acetic acid.

shows the plots of the iron added *vs.* the linewidth of the peak. As shown in this figure, the slope for methylamine obtained from the plot is much larger than that for acetic acid.

We define the k value which is the linewidth normalized by the concentration of ferritin added and represents the line broadening of each peak. The observed linewidth ΔH_{obs} may be given as

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

where ΔH_0 is the linewidth observed in the absence of ferritin and ΔH_p is a contribution from ferritin. When exchange between molecules which are under the influence of the iron core and those which are free in solution is fast enough, ΔH_p should increase linearly with the ferritin concentration (C) added. Therefore, $k(=\Delta H_p/C)$ should be constant, and

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

The k value for methylamine is 8, whereas that for acetic acid is 6×10^{-2}; the former is two orders of magnitude larger than the latter. In view of the fact that at pH 7.4 methylamine and acetic acid exist as cation and anion, respectively, it appears that this large difference is caused by the difference in interaction between each of these probes and the negative charge of the phosphate group on the surface of iron core.[10] In fact, when ferritin containing less phosphate (phosphate : iron ratio (P/Fe) 0.08) is used, the k value of methylamine is 2.2, which is one fourth of that for ferritin with a larger P/Fe ratio of 0.10. This suggests that the k value is a sensitive parameter for the difference of the content of the phosphate of ferritin, and that the NMR probe method is useful to investigate the phosphate of ferritin.

The k *Values for Various Probes. Effects of the Charge and Size of the Probes*: The interaction of various probes with ferritin were investigated systematically. The probes which have the amino, hydroxyl, and carboxyl groups were used as representatives of positively charged, neutral and negatively charged probes, respectively. In addition, probe molecules which have the methyl and *t*-butyl groups were compared to study effects of the size of the probes. Probes with aromatic rings were also used to investigate the effect of the aromatic ring. Results are shown at two different pH values, 7.4 and 3.2 in Figs. 2A and 2B, respectively, where abscissa

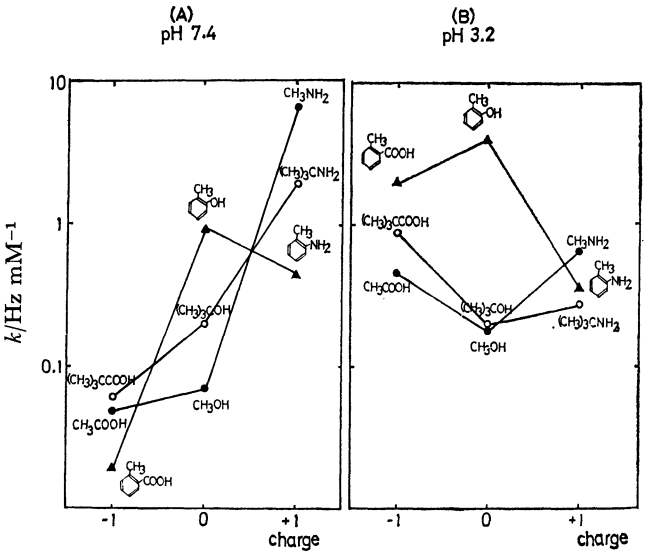

Fig. 2. The k values for probes with the amino, hydroxyl, and carboxyl groups. (A): pH 7.4, (B): pH 3.2.

represents the number of the charge of the probes used. The k values for neutral probes having the hydroxyl groups were about 0.1. The results for probes with the aromatic ring is quite different from those with the amino or carboxyl groups. It should also be noted that the k value for *o*-cresol is abnormally large and that *o*-toluidine is small compared with those of methylamine and *t*-butylamine.

At pH 3.2, the pattern of each curve in Fig. 2B

becomes quite different from that observed at pH 7.4; the k values for probes with the carboxyl group are much larger, whereas those for methylamine and t-butylamine are much smaller. By contrast, the k values for the neutral probes with the hydroxyl group remain constant. When the carboxyl groups of the probes lose their charges at low pH, electrostatic repulsion should decrease. Since the neutral probe has no charge, it is quite conceivable that the k value is not influenced by the change of pH.

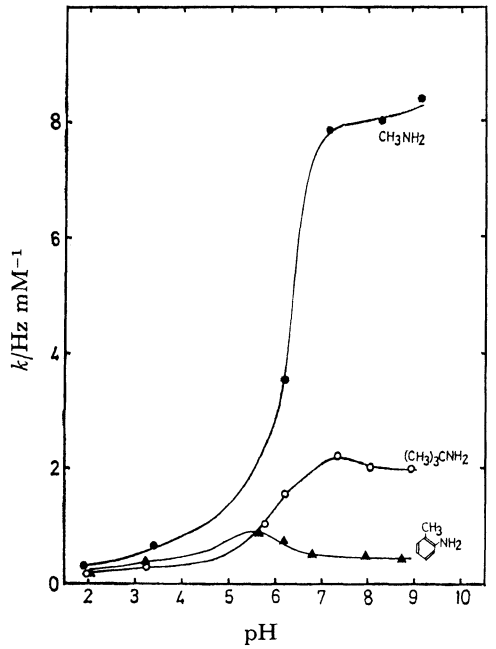

Fig. 3. The pH dependence of the k values for probes with the amino groups.
●: Methylamine, ○: t-butylamine, ▲: o-toluidine.

The pH *Dependence of the* k *Values. Positively Charged Probes with the Amino Group*: The pH dependence of the k values for various probes was investigated over a wide pH range in order to discuss in detail the results obtained above. The pH dependence of the k values for probes with the amino group is shown in Fig. 3. The pK_a of the phosphate in the iron core may be estimated from this curve.[10] It is seen in Fig. 3 that the k value for t-butylamine is one fourth of that for methylamine. The curve for o-toluidine has a maximum at pH 5—6. The charge of the amino group of o-toluidine with a pK_a of the amino group of 4.5 is lost at high pH; at the same time, the phosphate dissociates from the monoanion to the dianion in this pH range, suggesting that the maximum is due to a superposition of these two effects.

Neutral Probes with the Hydroxyl Groups: The pH dependence of the k values for methanol, t-butyl alcohol, and o-cresol is shown in Fig. 4. The k values for methanol and t-butyl alcohol are independent of pH. By contrast, the k value for o-cresol is much larger than those for methanol and t-butyl alcohol and has a maximum at low pH.

Negatively Charged Probes with the Carboxyl Group: As

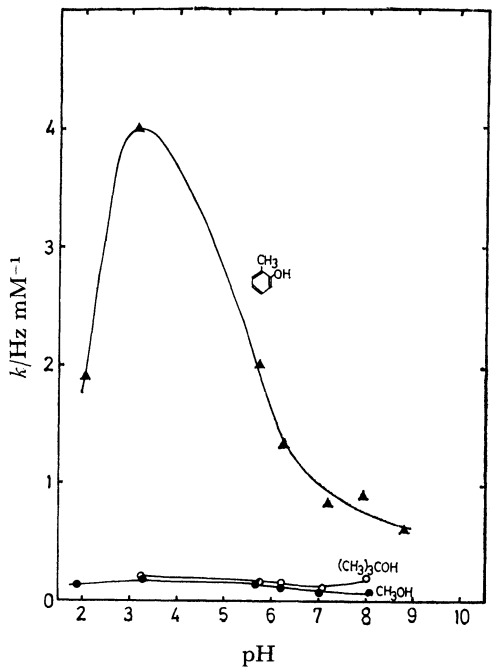

Fig. 4. The pH dependence of the k values for probes with the hydroxyl groups.
●: Methanol, ○: t-butyl alcohol, ▲: o-cresol.

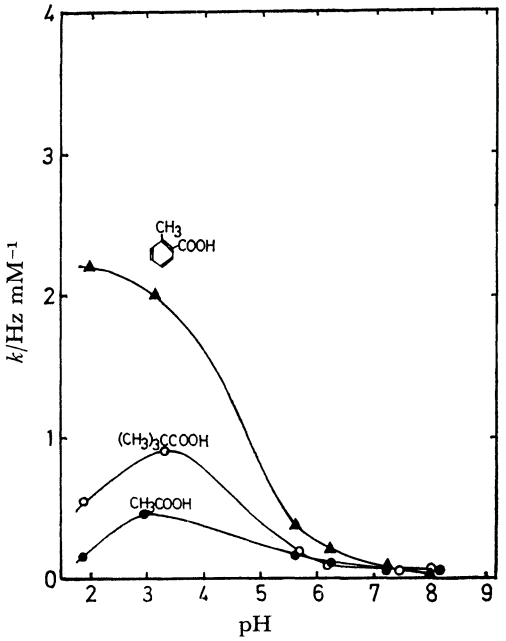

Fig. 5. The pH dependence of the k values for probes with the carboxyl groups.
●: Acetic acid, ○: pivalic acid, ▲: o-toluic acid.

Fig. 5 shows, the k values are quite small at pH 5 and increase below pH 5.

The affinity of Li^+, Na^+, *and* K^+ *with Ferritin.* Silk and Breslow[13] reported the effect of KCl and NaCl on the pH at which ferritin or apoferritin have no net charge, and suggested that K^+ ion binds with ferritin strongly. In the following we will show the usefulness of the NMR probe method in investigating interactions

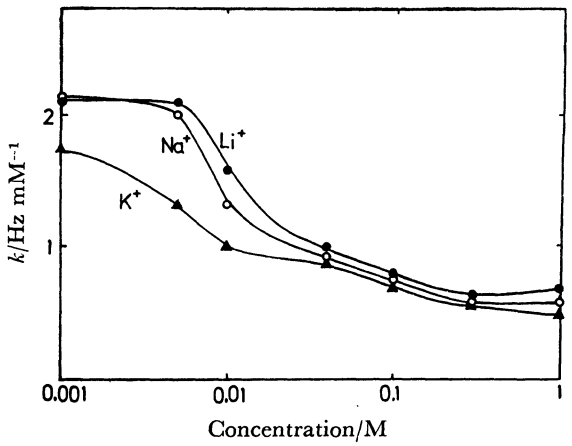

Fig. 6. The k values of t-butylamine as a function of the logarithmic concentration of Li^+, Na^+, and K^+ ions.
●: Li^+, ○: Na^+, ▲: K^+.

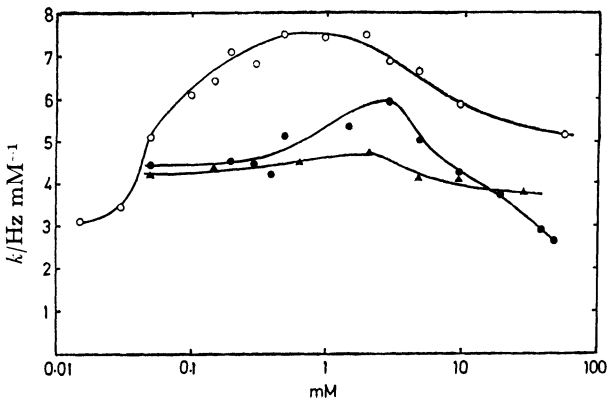

Fig. 7. The k values for t-butylamine as a function of the logarithmic concentrations of chelating agents.
▲: Acetic acid, ●: Citric acid, ○: EDTA.

between ferritin and various kinds of ions, alkali cations being taken as an example. In Fig. 6, the k values for t-butylamine were plotted against the logarithmic concentration of each of the alkali ions. From a decrease in the k value for t-butylamine, it may be concluded that the interaction of t-butylamine with ferritin becomes weak with the increasing concentration of the cations added. When the concentration of the cations is high, there is little difference among k values for the three kinds of cations. In the concentration range below 10 mM, the k values for Na^+ and Li^+ become identical and equal to those observed in the absence of these ions (k=2.1). This result suggests that, in this concentration range of the cations, the broadening of linewidth is not affected by these ions. However, in the case of K^+ ion, the k value was still small even at a quite low concentration of 1 mM. This appears to indicate that the affinity of K^+ to ferritin is much stronger than those of Na^+ and Li^+.

The Interaction of Chelating Agents with Ferritin. It is well-known that chelating agents, as well as reducing agents, are capable of releasing iron from ferritin.[1,2] It has been reported that the rate, at which iron is released from ferritin by chelating agents, is generally quite slow; for example, only a small amount of iron can be released from ferritin by EDTA.[14] The NMR probe method may be used to investigate whether or not the poor iron releasing ability of EDTA is due to a weak interaction between EDTA and ferritin. In the present experiment, we use EDTA, citric acid, and acetic acid with four (pK_1 2.0, pK_2 2.67, pK_3 6.16, pK_4 10.26), three (pK_1 3.09, pK_2 5.49, pK_3 6.15), and one (pK 4.67) carboxyl groups, respectively. As expected from the negative charges which all these molecules have, the k values are quite small. Therefore, the interaction between the chelating agents and ferritin was investigated by using more sensitive second probes such as t-butylamine as described previously. The results are shown in Fig. 7. The k values for t-butylamine are plotted against the logarithmic concentrations of the chelating agents. The k value for acetic acid remains almost constant in the whole concentration range examined, whereas the curve for citric acid has a maximum at about a concentration of 3 mM and the curve for EDTA has a large value in a wide concentration range 0.03 to 10 mM. These results suggest that acetic acid which has no chelating ability does not interact with ferritin, whereas citric acid interacts with ferritin strongly in the narrow concentration range 1 to 10 mM and EDTA interacts more strongly in a much wider concentration range.

Discussion

Interaction of the Probe Molecules with the Phosphate on the Iron core of Ferritin. In the previous paper[10] we have suggested that the probe molecules interact with ferritin through the phosphate on the iron core. In order to confirm this, we compare interaction on the probe molecules with two types of ferritin, ferritin I (Fe/protein ratio 0.20; P/Fe ratio 0.10) and ferritin II (Fe/protein ratio 0.30; P/Fe ratio 0.08). Ferritin II contains more iron and less phosphate than ferritin I.

We first consider the broadening of neutral probes (for example, solvent water) in a solution which contains each of these two types of ferritin. The k values for ferritins I and II are designated as k_I and k_{II}, respectively. It was observed that at the same iron concentrations, the ratio k_I/k_{II} for neutral probes is 1.1, whereas k_I/k_{II} for positively charged probes is 3—5.

The broadening of the linewidths may be assumed to be proportional to the exposed area of the iron core which probe molecules can freely contact with. This area may be calculated as follows: The size and surface area of iron core are estimated using Fe/protein ratio (the number of iron atoms per molecule) and X-ray data (the size of unit cell)[15] on the basis of the assumption that each ferritin molecule contains one spherical iron core. At a given amount of iron, *i.e.*, at the same iron concentration, the number of iron core contained in a solution increases and the product of the number and the surface area of one iron core (total surface area) increases as the particle size decreases. Therefore, the surface area increases as the size of iron core (iron content of ferritin) decreases. Figure 8 shows the calculated surface area of iron core as a function of the

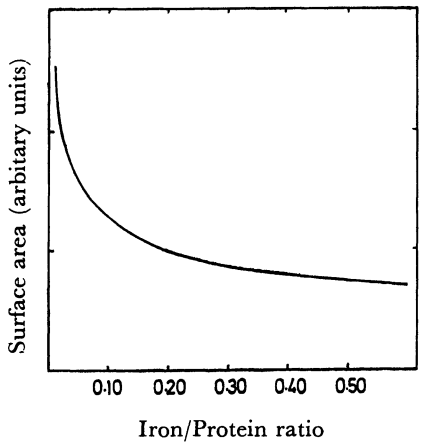

Fig. 8. The surface area of iron core as a function of the iron content of ferritin.

iron content of ferritin. This means that the linewidth should decrease as the iron content increases. This result is consistent with the report by Hoy *et al.*,[16,17] who reported that the rate of iron release from ferritin is closely related to the iron content.

In the above model, the ratio of the surface area of the iron core of ferritin I to that of ferritin II is about 1.1. This is consistent with the observation for the k values of the neutral probes. In view of these results, it may be concluded that the linewidth of the neutral probes is influenced only by the size of the iron core and not by the presence of the phosphate.

However, the line broadening for the positively charged probes in a ferritin I solution is quite different from that in a ferritin II solution. As mentioned above, the k_I/k_{II} ratio for the two solutions is 3—5. The large difference in k for the charged probes appears to be caused by the difference of the phosphate content. If the phosphate exists exclusively on the surface of the iron core,[4,5] the surface charge density of the phosphate for ferritin I should be larger than that for ferritin II. This would mean that the positively charged probe in the ferritin I solution is attracted to the iron core more strongly than in a ferritin II solution, and the k value for the ferritin I solution becomes larger than that for the ferritin II solutions. Since the protein part of ferritin I is presumably the same as that of ferritin II, it is likely that these differences are caused most probably by the phosphate on the iron core.

The Affinity of Li^+, Na^+, and K^+ with the Phosphate of Ferritin. The reason that the k values for t-butylamine decrease when the concentration of alkali ions becomes higher than 10 mM in Fig. 6 may be considered as follows: The alkali ions as well as t-butylamine have positive charges at pH$<$10 and presumably attracted to the negative charge of the phosphate of the iron core of ferritin. When the concentrations of the Li^+, Na^+, and K^+ ions are higher than that of t-butylamine (10 mM), it is probable that the alkali ions neutralize the negative charge of the phosphate; this would weaken the interaction of t-butylamine with ferritin and result in the narrowing of the linewidth of t-butylamine. On the other hand, when the concentration of alkali ions is lower than that of t-butylamine, the interaction of t-butylamine with the phosphate of ferritin would dominate and the linewidth of t-butylamine remain unchanged.

Interaction between Chelating Agents and Ferritin. Several chelating agents with the carboxyl groups were used to investigate how these molecules interact with ferritin. As mentioned previously, these molecules are subject to strong repulsing force from the negative charge of the phosphate on the core of ferritin and therefore should be insensitive as NMR probe. Thus, t-butylamine which is a sensitive NMR probe is added to the solutions, and the interaction between chelating agents and ferritin was investigated through the linewidth of t-butylamine. In this experiment, the linewidth of t-butylamine in a ferritin solution increases when the chelating agents are added. Possible reasons for the broadening of the linewidth of t-butylamine are as follows: 1) As mentioned previously, when the concentration of alkali ions increases, the negative charge of the phosphate of the iron core of ferritin is neutralized and the linewidth of t-butylamine decreases at a high concentration range of alkali ions as shown in Fig. 6. In the present experiment, this seems to be one of the reasons that each k value of EDTA, citric acid and acetic acid decreases in a high concentration range as shown in Fig. 7. 2) The negative charge of the chelating agents would cancel the positive charge of t-butylamine, resulting in a decrease in the k value of t-butylamine. 3) It is possible that the chelating agents strongly interact with ferritin and bind to the iron core of ferritin. The number of negative charges per ferritin molecule increases when the negatively charged chelating agents bind to the iron core of ferritin. This would also increase the k value of t-butylamine.

Conclusion

It has been shown that the NMR probe method is useful in obtaining information about ferritin and, in particular, the mode of interaction between the small molecules and ferritin. It was suggested that this interaction was determined primarily by the electrostatic repulsion and attraction which arise from the negative charge of the phosphate on the iron core of ferritin. It was also concluded that K^+ ion interacts strongly with the phosphate of the iron core even below a concentration of 1 mM and that EDTA binds strongly with ferritin in a wide concentration range 0.1—10 mM.

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