Permeation of small molecules into the cavity of ferritin as revealed by proton nuclear magnetic resonance relaxation

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The NMR relaxation technique was used to investigate the permeation of molecules into the cavity of ferritin. Spin-lattice relaxation times in the rotating frame of various probe molecules were measured for solutions of recombinant horse L-apoferritin without iron and horse spleen apoferritin with very small amounts

of ferric ions. The results show that molecules larger than the size of the ferritin channels can pass through the channels into the ferritin interior, and that the maximum size of molecules for the permeation is smaller than maltotriose.

INTRODUCTION

Ferritin is the main eukaryotic iron storage protein. It consists of 24 subunits [1] that surround an inorganic iron core containing up to 4500 iron atoms. This protein performs the two important specific biological functions of storing excess cellular iron and releasing it to cellular processes as needed [2]. The protein shell (apoferritin) is about 23 Å thick with an internal diameter of 76 Å. Two types of channels (three- and four-fold channels), which are 3–4 Å in diameter, penetrate the protein shell and serve as pathways between the exterior and interior of the shell [1].

The protein shell of ferritin is clearly permeable to iron, which can be transferred into and out of the protein interior. The permeation of other molecules such as reductants and iron chelators through the channels is proposed by a number of kinetic experiments of iron deposition or release [3–5]. In contrast, the permeation of reductants and chelators larger than the channels, such as FMN, FAD, methylviologen, and bipyridine, was questioned by some investigators [6,7]. Thus, there is no general agreement regarding the permeation of molecules larger than the channel size.

Paramagnetic agents can relax nuclear spins of molecules in their proximity [8,9]. If paramagnetic agents are specifically attached inside the apoferritin cavity so that they can only affect molecules (probes) within the cavity, then the change of the relaxation times of the probe molecules can give direct evidence for their permeation. The iron core in holoferritin is a natural paramagnetic agent. Thus, the phosphate on the iron core of holoferritin was studied by a proton NMR probe method [10]. However, the iron core contains too many ferric ions (2000–3000 for commercial holoferritin) to serve our purpose because its effect on the nuclear spin relaxation of molecules (i.e. outer sphere relaxation effect) extends out to several ferritin radii [9].

In this investigation, we use an NMR relaxation technique to investigate the permeation of probe molecules. A small amount of ferric ions was incorporated into the interior of horse spleen apoferritin to serve as a paramagnetic relaxation agent. Relaxation time measurements for a series of probe molecules in solutions of recombinant horse L-apoferritin (rhLAF) without iron and horse spleen apoferritin containing very small amounts of ferric ions show that molecules as large as maltose can permeate into the ferritin interior.

MATERIALS AND METHODS

All chemicals were reagent grade and used without further purification. Heavy water ($^2\text{H}_2\text{O}$) containing 99.9 atom % ^2H was purchased from Aldrich Chemical Company. Horse spleen apoferritin was purchased from Sigma Chemical Company. Maltose, D-glucose, and polyethylene glycol (M_r 7500) were purchased from Wako Pure Chemical Industries Ltd. Maltotriose, maltotetraose, and maltopentaose were purchased from Hayashibara Biochemical Laboratories, Inc. rhLAF was prepared as in [11].

To examine the permeation of molecules into the cavity of ferritin by NMR relaxation, the probe molecules used should be soluble in water and they should not cause the release of ferric ions from the interior of ferritin through chelation or reduction. Therefore, molecules with different sizes such as water, DMSO, glucose, maltose, maltotriose, maltotetraose, maltopentaose, and poly(ethylene glycol) (PEG, M_r 7500) were chosen. Their approximate sizes are listed in Table 1.

Preparation of horse spleen apoferritin with a small amount of ferric ions

Ferrous ammonium sulphate was used as the iron source in the presence of oxygen as oxidant. A small amount of iron, corresponding to about 24 Fe^{2+} per protein, was added to a 1 mg/ml horse spleen apoferritin solution in 20 mM Mops buffer at pH 6.5. After incubation at 37 °C for 1 h, sodium citrate (at a final concentration corresponding to the iron added) was added to stop the reaction. Then, any non-specifically bound iron was removed by dialysis against a 20 mM Mops buffer, pH 6.5, containing 1 mM citrate for 24 h followed by dialysis against buffer alone [12]. The ferritin thus obtained is referred to as Fe-AF. The sample prepared in this way contained no free iron ions in the solution and no bound iron ions on the outer surface of the protein. The iron in Fe-AF probably exists in the ferritin cavity as a precursor of the ferrihydrite core crystallite at this low Fe/protein ratio as indicated by EXAFS studies [13]. The concentration of the protein was determined by the Lowry method [14]. The iron content of the Fe-AF was determined as the 2,2'-bipyridyl complex at 520 nm [15]. This revealed that there were about 16 iron atoms per molecule.

Table 1 Approximate sizes, $T_{1\rho}$ and $\Delta R_{1\rho}$ of probe molecules

The approximate sizes of probe molecules were estimated from bond lengths and angles. $T_{1\rho}$ was measured at 25 °C with a spin-locking field strength ω_1 4.17 kHz and a protein concentration of 8.4 mg/ml. The standard error for $T_{1\rho}$ was about 5%. $\Delta R_{1\rho}$ was calculated from eqn. (1).

Probes	Approximate length (Å)	Proton	$T_{1\rho}$ (s)		$\Delta R_{1\rho}$ (s ⁻¹)
			rhLAF	Fe-AF	
Water	2		2.32	0.77	0.87
DMSO	5		4.70	1.70	0.38
Glucose	7	H ₁ (α)	2.82	1.16	0.51
		H ₁ (β)	1.88	1.01	0.46
Maltose	13	H ₁ (α)	1.93	1.08	0.41
		H ₁ (β)	1.04	0.72	0.43
		H ₁ ′	0.79	0.60	0.40
Maltotriose	20	H ₁ (α)	1.55	1.10	0.26
		H ₁ (β)	0.77	0.66	0.22
		H ₁ ′, H ₁ ″	0.56	0.50	0.21
Maltotetraose	28	H ₁ (α)	1.32	1.08	0.17
		H ₁ (β)	0.73	0.64	0.19
		H ₁ ′, H ₁ ″	0.50	0.46	0.17
Maltopentaose	34	H ₁ (α)	1.11	0.93	0.17
		H ₁ (β)	0.69	0.60	0.22
		H ₁ ′, H ₁ ″	0.43	0.40	0.17
PEG	> 34		0.61	0.54	0.21

NMR sample preparation and methodology

The water in the solutions of the rhLAF and Fe-AF was replaced by heavy water three times using ultrafiltration (YM100, Amicon). Samples used for relaxation measurements were prepared by adding ferritin (rhLAF or Fe-AF) and probe solutions to heavy water. The final concentrations of probe molecules were 10 mM in all NMR measurements except for PEG, which was 0.05 mM. The final concentrations of rhLAF and Fe-AF were 8.4 mg/ml in all measurements.

The ¹H-NMR spectra were obtained with an NMR spectrometer (JNM-A500, JEOL) operating at 500 MHz. In this study, the spin-lattice relaxation time in rotating frame ($T_{1\rho}$) was chosen to examine the permeation because this does not suffer from the modulation of scalar coupling as does transverse relaxation time (T_2). $T_{1\rho}$ was measured by the spin-locking pulse sequence, $90^\circ_x - t(\text{SL})_y - \text{Acq}$, where (SL) denotes spin-locking and t is the spin-locking time. The carrier was placed on the resonance frequency of the proton of interest to avoid the offset effect. The interval between subsequent experiments was greater than five times T_1 to assure complete spin equilibrium. The data were fitted using a non-linear least-square program. All relaxation experiments were repeated three times and the results were averaged.

Proton relaxation of probe molecules in Fe-AF solution

In the Fe-AF solution, the observed relaxation rate of the probe molecules can be attributed to interactions within the probe molecule itself, to probe-Fe³⁺ interactions, and perhaps to probe-protein interactions. The protein structure of rhLAF should be very similar to that of Fe-AF [16,17]. The rhLAF contains no iron ions, so the difference between the relaxation rates of probe molecules in the rhLAF and Fe-AF solutions

[R_i (in rhLAF) and R_i (in Fe-AF)], where $R_i = 1/T_i$ and $i = 1, 2$, and 1ρ] is due exclusively to the paramagnetic contribution of the probe-Fe³⁺ interactions, as follows.

$$\Delta R_i = R_i(\text{in Fe-AF}) - R_i(\text{in rhLAF}) \quad (1)$$

This paramagnetic contribution can be described by inner sphere theory, outer sphere theory, or by a two-site exchange model as explained below.

Inner sphere relaxation arising from probe molecules bound to paramagnetic particles with sufficiently long lifetimes can be described by the Solomon-Bloembergen equations [8]. This relaxation is inversely proportional to the sixth power of the distance between the electron and the nucleus. Outer sphere relaxation arises from the relative diffusion of the probe molecules in the vicinity of the paramagnetic particles [9]. The contribution of outer sphere relaxation to $1/T_{1\rho}$ of the probe molecules is as follows.

$$\frac{1}{T_{1\rho}} = \frac{32\pi}{405} \gamma_1^2 \mu^2 \frac{N_A}{1000 RD} [6.5j(\omega_s \tau_R) + 1.5j(\omega_1 \tau_R) + 2j(\omega_1 \tau_R)] \quad (2)$$

Here γ_1 is the nuclear gyromagnetic ratio, μ is the magnetic moment of each particle, N_A is Avogadro's number, $[M]$ is the molar concentration of the particles, R is the closest distance between the particle centre and the probe molecule centre, D is the relative diffusion coefficient for the particles and probe molecules, ω_1 and ω_s are the proton and electron Larmor frequencies respectively, ω_1 is the spin-locking field strength used in $T_{1\rho}$ measurements, τ_R is the correlation time for translational diffusion (given by $\tau_R = R^2/D$), and $j(\omega)$ is the spectral density function, given as follows.

$$j(\omega) = \text{Re} \left\{ \frac{1 + 0.25(i\omega\tau_R)^{\frac{1}{2}}}{1 + (i\omega\tau_R)^{\frac{1}{2}} + (4/9)(i\omega\tau_R) + (1/9)(i\omega\tau_R)^{\frac{3}{2}}} \right\}$$

Under the condition of $\omega_1 \tau_R \ll 1$, the paramagnetic contribution to $T_{1\rho}$ is the same as that to T_2 .

An alternative simple approach to describe the relaxation in this system is to consider the exchange of molecules between the two environments [18]. For a well-defined two-site model, the relaxation time $T_{1\rho}$ is given as follows:

$$\frac{1}{T_{1\rho}} = \frac{x_a}{T_{1\rho}(a)} + \frac{x_b}{T_{1\rho}(b) + \tau_M} + \frac{x_a x_b (\Delta\omega)^2 \tau_M}{1 + (\omega_1 \tau_M)^2} \quad (3)$$

Here x_a and x_b are the mole fractions of probe molecules at sites A and B respectively, $T_{1\rho}(a)$ and $T_{1\rho}(b)$ are the $T_{1\rho}$ values of the probe molecules at sites A and B respectively, $\Delta\omega$ is the difference between the proton resonance frequencies at sites A and B, and τ_M is the exchange time between the two sites.

RESULTS

In aqueous solution, both the α and β forms of glucose and oligoglucose exist (Figure 1). The $T_{1\rho}$ values of the residual protons of heavy water, the protons of DMSO and PEG, the H₁ proton of glucose, the H₁ and H₁′ protons of maltose, and the H₁, H₁′, and H₁″ protons of oligoglucose were measured. For oligoglucose, the signals of the H₁′ and H₁″ protons were overlapped. The $T_{1\rho}$ values of the probes in the rhLAF and Fe-AF solutions measured at ω_1 4.17 kHz and 25 °C are shown in Table 1. To examine the binding of probe molecules on the outer surface of ferritin, at constant concentrations of rhLAF and Fe-AF (8.4 mg/ml), we measured the dependence of the $T_{1\rho}$ values on the concentrations of glucose, maltose and maltotetraose at ω_1 4.17 kHz and 25 °C. For both rhLAF and Fe-AF, no such dependence was found in the range 0.5–10 mM probes. To examine the permeation rate of the probe molecules, we measured

Figure 1 Diagram of oligoglucose

Table 2 Dependence of $\Delta R_{1\rho}$ of residual proton of heavy water, DMSO and $H_1(\alpha)$ proton of glucose on spin-locking field strength ω_1

$T_{1\rho}$ was measured at 25 °C.

Probe	ω_1 (kHz)	$T_{1\rho}$ (s)		$\Delta R_{1\rho}$ (s ⁻¹)
		rhLAF	Fe-AF	
Water	4.17	2.32	0.77	0.87
	2.06	2.28	0.75	0.89
	1.42	2.19	0.74	0.89
	0.54	2.02	0.70	0.93
	0.24	1.86	0.67	0.95
	0.16	1.78	0.66	0.95
DMSO	4.17	4.70	1.70	0.38
	2.06	4.73	1.72	0.37
	1.11	3.95	1.59	0.38
	0.54	3.80	1.55	0.38
Glucose	4.17	2.82	1.16	0.51
	2.06	3.24	1.19	0.53
	1.11	3.60	1.21	0.55
	0.54	4.03	1.27	0.54

Table 3 Dependence of $\Delta R_{1\rho}$ of residual proton of heavy water, DMSO and $H_1(\alpha)$ proton of glucose on temperature

$T_{1\rho}$ was measured at a spin-locking field strength ω_1 4.17 kHz.

Probe	T (°C)	$T_{1\rho}$ (s)		$\Delta R_{1\rho}$ (s ⁻¹)
		rhLAF	Fe-AF	
Water	25.0	2.32	0.77	0.87
	35.0	2.24	0.80	0.80
	45.0	2.29	0.83	0.77
DMSO	25.0	4.70	1.70	0.38
	35.0	5.51	1.95	0.33
	45.0	6.49	2.16	0.31
Glucose	25.0	2.82	1.16	0.51
	35.0	3.48	1.31	0.48
	45.0	4.49	1.45	0.47

the dependence of the $T_{1\rho}$ values on spin-locking field strength and temperature. The results are listed in Tables 2 and 3 respectively. The standard error of the $T_{1\rho}$ values is about 5%.

The values of $\Delta R_{1\rho}$ are also shown in the Tables. The decrease of $T_{1\rho}$ for protons H_1 , H_1' , and H_1'' with increasing ω_1 arises from the averaging of the $T_{1\rho}$ values of all protons in the same coupling network when ω_1 is much larger than the chemical shift difference between the coupling protons [19].

DISCUSSION

The primary purpose of this investigation was to show whether molecules larger than the nominal size of the ferritin channels can pass through the channels into the protein interior. If probe molecules can bind to slowly tumbling macromolecules and exchange between free and bound sites, the NMR relaxation rate of probe protons should be inversely proportional to the concentration of the probe molecules [20]. Therefore, our results indicate that glucose, maltose and maltotetraose bind on the surfaces of neither rhLAF nor Fe-AF. The other interpretation is that a very small portion of probe molecules strongly bind on the surfaces and, therefore, no exchange between bound and free sites occurs. In both the cases, $\Delta R_{1\rho}$ does not result from binding of probe molecules on the ferritin surfaces.

First, let us suppose that probe molecules do not penetrate into the ferritin cavity. Here, the probe molecules are so far from the ferric ions in Fe-AF (at least 23 Å) that the proton-Fe³⁺ inner sphere interactions are much weaker than the proton-proton interactions inside the probe molecules. Therefore, the contribution of inner sphere relaxation would be negligible. Because $\omega_1\tau_R \gg 1$ and $\omega_1\tau_R \ll 1$ ($\tau_R \approx 20$ ns for water), the contribution of outer sphere relaxation should be proportional to $1/RD$ [eqn. (2), $D = kT/6\pi\eta r$ and $R = r + a$, where r and a are the radii of ferritin and the probe molecule respectively]. Thus, the larger the molecule, the larger the paramagnetic contribution. From Table 1, however, we find that $\Delta R_{1\rho}$ decreases with increasing size of the probe molecule. This result does not agree with a model of no penetration.

Next, let us consider that small probe molecules can penetrate into the ferritin cavity. If the dominant relaxation mechanism in the cavity of Fe-AF is an inner sphere component, then the relaxation rate $1/T_{1\rho}$ of probe molecules in the cavity must be much larger than that outside ferritin. Even if the main relaxation mechanism in the cavity is outer sphere relaxation, probe molecules (e.g. 5 Å radius) in the cavity contribute more than 90% of the total relaxation rate, provided that the closest distances between ferric ions and probe are 5 Å when the probe is in the cavity and 65 Å when the probe is outside the ferritin [eqn. (2)]. We can see that the protein shell of Fe-AF makes the environment in the cavity much different to that outside the ferritin. Because of these two different sites (i.e. site A is outside the protein shell and site B is inside the protein cavity) eqn. (3) is suitable for describing the relaxation of probes in the Fe-AF solution. Here, the results shown in Table 1 can be interpreted by using eqn. (3) in terms of various values of x_b and $T_{1\rho}(b) + \tau_M$ for different probes.

Obviously, the PEG molecule (M_r 7500) cannot penetrate into the ferritin cavity [i.e. $x_b = 0$ in eqn. (3)]. The value of $\Delta R_{1\rho}$ of PEG should be the paramagnetic contribution of ferric ions in Fe-AF to the probe molecules outside the protein shell. For maltotriose, maltotetraose and maltopentaose, their values of $\Delta R_{1\rho}$ are almost the same as that for PEG. For small molecules such as water, DMSO, glucose and maltose, however, their values of $\Delta R_{1\rho}$ are significantly larger (Table 1). Therefore, we conclude that molecules that have a size similar to that of maltose can permeate into the ferritin cavity.

No dependence of $T_{1\rho}$ on the concentrations of glucose and maltose in an Fe-AF solution suggests that the mole fraction of

the probe in site B (x_b , in the cavity) does not change with changing total probe concentration in the range 0.5–10 mM [eqn. (3)]. The volume of one ferritin cavity is about 2.3×10^{-22} litre. Even if the average concentration of probe molecules in the cavity is as large as that in the solution, there are only about 1.4 probe molecules in one cavity in a ferritin solution containing 10 mM probes. So, it is reasonable that the average concentration of glucose or maltose in the ferritin cavity increases with increasing total probe concentration over the range 0.5–10 mM.

The measurement of $\Delta R_{1\rho}$ at different ω_1 values can potentially allow us to obtain the exchange rates of probe molecules between the two sites. We, however, did not find a clear dependence of $\Delta R_{1\rho}$ on ω_1 values from 0.16 to 4.17 kHz for water and from 0.54 to 4.17 kHz for DMSO and glucose (Table 2). This might occur because the difference between the average chemical shifts at site A and site B is too small [i.e. $\Delta\omega\tau_M \ll 1$ in eqn. (3)]. Another possibility is that $\omega_1\tau_M \ll 1$.

The relaxation times $T_{1\rho}(a)$ and $T_{1\rho}(b)$ increase and the exchange time τ_M decreases when the temperature is increased. The values of $\Delta R_{1\rho}$ of water, DMSO and glucose tend to decrease with increasing temperature (Table 3). This suggests that τ_M is shorter than $T_{1\rho}(b)$ [eqn. (3)]. An estimation of the maximum amount of water in the cavity (76 Å diameter) gives a value of 0.3 g/g of protein for apoferritin. Thus, the mole fraction of water in the cavity is about 0.0025 for a protein concentration of 8.4 mg/ml. Neglecting the last term in eqn. (3) and the paramagnetic contribution to $\Delta R_{1\rho}$ from the water in the exterior of protein, $T_{1\rho}(b) + \tau_M$ is roughly equal to $x_b/\Delta R_{1\rho}$ [eqn. (3), i.e. about 3×10^{-3} s at 25 °C]. Our rough estimation indicates that τ_M for water is shorter than 3×10^{-3} s. Supposing that the average concentration of probe molecules in the cavity is the same as that in the solution, similarly, we find that the values of τ_M for glucose, maltose and DMSO are shorter than 5×10^{-3} , 6×10^{-3} , and 7×10^{-3} s.

Although Jacobs et al. found no evidence for the permeation of molecules such as FMN, methylviologen, and 2,2'-bipyridine into ferritin, they did not exclude the possibility that these molecules diffused too fast to be measured by their methods [6]. Our conclusion that molecules with a size similar to that of maltose can permeate into the ferritin cavity is consistent with the results of several previous studies [3–5,21]. For example, a neutron-scattering examination of apoferritin indicates that glucose diffused through the shell at a rate that was too fast to be measured. Jones et al. suggest that free FMN enters the

ferritin interior, but that polymer-bound FMN was prevented from doing so. The permeation of probe molecules larger than the channel size implies that the protein shell is sufficiently flexible. The flexibility should be further investigated by directly studying the protein itself.

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