In Vivo and In Vitro Studies of Hafnium-Binding to Rat Serum Transferrin

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ABSTRACT

The binding of hafnium to rat serum transferrin was studied using the time differential perturbed angular correlation (TDPAC) technique. Hafnium is interesting as a toxic metal binding to transferrin because it behaves metabolically similarly to plutonium. The isotope ^{181}Hf offers favorable access to the TDPAC-method. Samples were prepared in vivo by intravenous injection of Hf–NTA, Hf–citrate, and Hf–oxalate solutions, respectively, into Sprague–Dawley rats and in vitro by adding Hf-NTA solution to fresh rat serum. In both cases two specific electric quadrupole interactions were observed, which correspond to two well-defined binding configurations. They may be attributed to the N-terminal and the C-terminal binding site in the transferrin molecule. The ^{181}Hf-distribution between these two binding states depends on pH, salt and hafnium concentrations, temperature, and incubation time. With a fast TDPAC-setup of four BaF_2-detectors a time resolution of about 600 ps could be achieved. The specific binding configurations of ^{181}Hf and the comparatively slow relaxation times lead to spectra of considerable accuracy.

INTRODUCTION

It is well established that serum transferrin is the essential transport protein for metabolic iron in mammals. In addition, iron transferrin also binds a wide variety of

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Journal of Inorganic Biochemistry **27**, 255–270 (1986)

0162-0134/86/$3.50

other metals. The simultaneous binding of a synergistic anion (e.g., bicarbonate) is required for a stable metal–transferrin complex.

The transferrin molecule is a single chain polypeptide (molecular weight ~80,000) which is characterized by a bilobal structure. Each of the two domains (called N- and C-domain, respectively) contains one metal-binding site. By employing chemical and spectroscopic methods, differences between the two binding sites were found (see recent reviews in [1–3]). It has been shown with iron that the two sites are not equally populated in the circulation [4]. Therefore differences in their biological function might exist. The distance between the two sites seems to be rather large (about 3.5 nm) [5]. The two domains of the protein should interact to account for the cooperativity of the metal binding, which has been observed in some cases (see e.g., [6]). There is evidence for conformational changes within the protein when metal ions are bound (cf. [2]).

The differences between the two domains of transferrin in terms of structure and chemical properties have been described frequently. The complete amino acid sequence of human transferrin is now available [7]. An important result is that the C-terminal half of transferrin is an imperfect repeat of the N-terminal domain. Homology between the amino acid sequences of the two domains for different transferrin species limits the number of amino acid residues which may be involved in the iron binding of transferrin. Taking into account all the available information models have been proposed for the way in which the metal-binding sites arise from the folding of the protein [2, 8].

Only a few studies have been performed so far concerning biological aspects of hafnium in general and its metabolic behavior in particular [9–11]. Recently it has been demonstrated [12] that under carefully defined conditions hafnium behaves similarly to plutonium in its metabolism and its interactions with biochemical ligands in cells and tissues. Chemically hafnium appears predominantly in the tetravalent state with eightfold coordination, the ionic radius $r_0 = 0.083$ nm being quite close to the plutonium(IV) ionic radius $r_0 = 0.086$ nm for, as a rule, sixfold coordinated ions [13].

Hafnium seems to be a suitable substitute for plutonium in biological studies with respect to the metal binding to transferrin. Handling of ^{181}Hf as a β-emitter is by far less restricted. A striking advantage of using hafnium as a probe nucleus lies in the fact that the isotope ^{181}Hf offers access to the spectroscopic method of perturbed angular correlations of gamma rays which is highly sensitive to oxidation states of the probe nuclei and to the ligand fields acting on them. Thus further knowledge of the microscopic structure of the metal-binding sites in the protein can be expected from the application of this method.

THE PERTURBED ANGULAR CORRELATION TECHNIQUE

The time differential perturbed angular correlation (TDPAC) technique is based on the observation of an anisotropic correlation between the emission directions of two successive gamma-radiations while the nucleus is exposed to an electromagnetic perturbation. With respect to other hyperfine interaction techniques it shares with the Mössbauer method the advantage of high specificity. In comparison to the latter, however, the requirements relating to the sample conditions are far less restrictive: Not only solid but also liquid and gaseous materials are accessible. Thus the method seems to be particularly promising for the investigation of biomolecules where these are to be studied in solution.

The isotope ^{181}Hf is very suitable for TDPAC studies. It decays by β-emission with a half life of 42.4 d into ^{181}Ta. With a branching of 93% the state with nuclear spin $I = 1/2$ is populated which deexcites via a γ(133 keV) $\rightarrow$ γ(482 keV) cascade to the ground state of ^{181}Ta with $I = 7/2$. During the lifetime of the intermediate state ($I = 5/2$, $t_{1/2} = 10.8$ ns) its nuclear quadrupole moment interacts with the electric field gradient (EFG) at the position of the nucleus. In general the ligand fields lead to an inhomogeneous field configuration which, in a classical sense, gives rise to a precession of the static angular correlation. In a quantum mechanical picture this corresponds to a periodical change of the m-substate population. It is the sensitivity of the observed spectra to the special electronic configuration in the immediate neighborhood which makes the method so attractive for studies of the binding state of the probe nucleus.

For samples without microscopic ordering, e.g., polycrystalline or dilute material, the correlation function is usually written as [14]

$$W(\theta, t) = \sum_{\nu} A_{\nu\nu} G_{\nu\nu}(t) P_{\nu} (\cos\theta), \qquad \nu \text{ even},$$

where the $A_{\nu\nu}$ are tabulated correlation coefficients that depend on the involved spins and the multipolarities of the emitted radiation only. P_{ν} ($\cos\theta$) are Legendre polynomials with θ being the angle between the two emission directions of the gamma quanta. If only terms with $\nu = 2$ are considered—an approximation that is sufficiently accurate in most cases—and for half-integer spin $I = 5/2$ the perturbation factor explicitly reads

$$G_{22}(t) = e^{-\lambda t} \sum_{k=0}^{3} s_{2k}(\eta) e^{-n_k(\eta)\omega_0 \delta t/2} e^{-(n_k(\eta)\omega_0 \sigma)^2/16 \ln 2} \cos(n_k(\eta)\omega_0 t),$$

where λ = the relaxation constant for a time-dependent interaction, ω_0 = the angular frequency, η = the asymmetry parameter which characterizes the deviation of the EFG configuration from axial symmetry, δ = the frequency distribution which is here assumed to be lorentzian, and σ = the time resolution of the TDPAC setup. The observed frequencies are $\omega_k = n_k(\eta)\omega_0$ (with $k = 1, 2, 3$). For an axially symmetric electric field gradient ($\eta = 0$) the frequencies ω_2 and ω_3 are harmonics of the fundamental frequency ω_1. This is no longer true for finite η values, where the coefficients $n_k(\eta)$ and $s_{2k}(\eta)$ have to be evaluated numerically (see, e.g., [15]).

Usually instead of the angular frequency ω_0, the so called quadrupole coupling constant is given:

$$\nu_Q = eQV_{zz}/h,$$

where e is the unit charge and Q the nuclear quadrupole moment. The relation between ω_0 and the quadrupole coupling constant for $I = 5/2$ is

$$\nu_Q = 10/(3\pi)\omega_0.$$

The absolute values of the electric field gradients are

$$|V_{zz}| = h\nu_Q/eQ,$$

$$|V_{yy}| = |V_{zz}|(1+\eta)/2,$$

$$|V_{xx}| = |V_{zz}|(1-\eta)/2,$$

with the asymmetry parameter $\eta = (V_{xx} - V_{yy})/V_{zz}$ and $|V_{zz}| \geqslant |V_{yy}| \geqslant |V_{xx}|$, i.e., $0 \leqslant \eta \leqslant 1$.

As will be pointed out later several different metal-binding conditions were observed for hafnium in transferrin. The perturbation factor for this case then reads

$$G_{22}^{\text{tot}}(t) = \sum_i f_i G_{22}^{(i)}(t).$$

The $G_{22}^{(i)}(t)$ are the perturbation factors as introduced above and f_i the fractions of the nuclei which have the electronic environment characteristic for the binding condition (i).

The measurements presented here were performed with a four detector slow-fast coincidence system. The 40 mm $\emptyset$ × 40 mm BaF_2 scintillators were directly coupled to fast quartz-window photomultipliers (Valvo XP 2020 Q). The coincidence spectra $N(\theta, t)$ were taken under fixed angles $\theta = 90°$ and $\theta = 180°$. To increase counting statistics, eight different spectra $N(\theta, t)$ were recorded simultaneously. As the correlation functions $W(\theta, t)$ are related to $N(\theta, t)$ the perturbation function $G_{22}(t)$ can be directly extracted from the data by forming the ratio

$$R(t) = 2\,\frac{N(180°, t) - N(90°, t)}{N(180°, t) + 2N(90°, t)} = A_{22}^{\text{eff}} G_{22}(t).$$

In all the spectra shown below this $R(t)$ ratio is plotted versus the time the perturbation is observed. The anisotropy coefficient A_{22} can be evaluated by taking into account the finite solid angle of the detectors and possible additional gamma-gamma cascades that cannot be separated from the relevant one. For the conditions given in this experiment $A_{22}^{\text{eff}} = -0.23$ was determined.

The time resolution parameter σ is of paramount importance. Poor time resolution leads to an increasing reduction of the amplitudes for high frequencies. A conventional NaI(Tl) detector system has a typical time resolution between 2 and 2.5 ns and therefore an upper detection limit of about 2 GHz. BaF_2 detectors which were introduced to TDPAC measurements by Baudry et al. [16] allow the determination of much higher frequencies. Using a set of four specially designed BaF_2 scintillators a time resolution of 600 ps was achieved for the gamma energies relevant to this study. With this improved system the asymmetry parameters of rather strong electric field gradients could be observed with high accuracy. For a comparison with earlier data see [17]. A more detailed description of the TDPAC apparatus will be given elsewhere [18].

MATERIALS AND METHODS

In vitro Preparations

Hafniumchloride solution in 2 *M* HCl was purchased from Amersham-Buchler. The specific activity was stated to be approximately 25 μCi ^{181}Hf per μg hafnium. One part of this solution was added to four parts of nitrilotriacetic acid (NTA) and then neutralized by an appropriate amount of sodium bicarbonate. As shown in previous studies [19, 20] hafnium then forms soluble complexes of Hf-NTA. Different amounts of Hf-NTA solution were added to samples of 1 ml freshly separated Sprague-Dawley rat serum.

The iron-binding capacity of this rat serum was controlled as part of the regular surveillance of the animal colony. Using commercial kits for the estimation of serum iron and iron-binding capacity (Boehringer, Mannheim, F.R.G.) a typical result observed with a group of 12 rats was 38 ± 4 nmol/ml for serum iron and 36 ± 5 nmol/ml for the unsaturated iron-binding capacity (UIBC) [21]. The percentage iron-saturation deduced from these results was 51 ± 4%. Therefore the rat serum samples contained approximately 3 mg or 37 nmol transferrin. Ratios of Hf-ions to the number of free binding sites in transferrin, K = [Hf]:[UIBC], between K = 1:15.6 and K = 1:3.1 were achieved.

Gel-filtration using a Sephacryl S-200 column was performed followed by an analysis of the transferrin containing fraction on DEAE cellulose to demonstrate that hafnium was almost completely bound to transferrin in rat serum [12] and human serum [22]. Comparative studies with ^{59}Fe and ^{181}Hf showed the identity of the elution profiles for the two nuclides. The ability of iron to prevent the binding of hafnium to iron-saturated serum or to release the radionuclide from its transferrin complex was studied using the methods described in [23]. Rat serum was labeled in vitro with ^{181}Hf or stable iron and analyzed by gel chromatography on Sephadex G-50. The results are shown in Table 1:

With increasing percent iron-saturation of the serum from 32% to 86% the fraction of ^{181}Hf bound to the protein was reduced approximately by a factor of 5; in the presence of excess iron virtually none of the ^{181}Hf was bound to protein.

The saturation of serum previously labeled with ^{181}Hf with iron resulted in the release of more than 98% of the bound hafnium.

Similar studies with human apotransferrin (Sigma, München, F.R.G.) showed that presaturation of the protein with iron reduced the ^{181}Hf-binding to less than 1% as compared to 92 ± 10% with the iron-free protein. Addition of iron to ^{181}Hf-labeled apotransferrin promoted the rapid release of the hafnium nuclide. These data all confirm that iron and hafnium are bound to the same specific binding sites on the transferrin molecule.

In Vivo Preparations

Sprague–Dawley rats were intravenously injected with solutions of Hf–NTA, Hf–oxalate, or Hf–citrate. Hf–NTA and Hf–oxalate were prepared as described above by

TABLE 1. The Influence of Iron on the Binding to Rat Serum Proteins[a]

Sample		% Iron saturation	% Total serum ^{181}Hf recovered in protein fraction from the Sephadex G-50 column
^{181}Hf-labeled rat serum	A	32	91 ± 3
	B	84	17 ± 4
	C	150	<2
Stable iron added to ^{181}Hf-labeled rat serum (A)		86	54 ± 12
		>200	<2

[a] Means (± SEM) of three analyses per point. Hafnium concentration in serum: 3 nmol/ml.

adding one part of hafnium to four parts of anion solution. For Hf-citrate a considerable surplus of citrate was necessary to guarantee the complexation of the metal. The serum taken 15 min after the Hf-NTA and Hf-oxalate injections contained about 16% of the injected activity of 200 μCi and 300 μCi, respectively. In the case of Hf-citrate injections 80% and 33% of the administered activity, respectively, were still present in the serum 12 and 60 min after injection. In terms of the hafnium concentration with respect to the UIBC of the transferrin these different retention values correspond to ratios between K = 1:60 and K = 1:7. Again, by gel-filtration methods it was demonstrated that more than 95% of the hafnium was bound to transferrin [12].

pH Value Adjustments

The injection solutions for the in vivo studies were adjusted to the physiological pH = 7.4. In one particular case, i.e., after Hf-oxalate injection the pH of the separated serum was also examined when the measurements were completed. The recorded value of 7.48 proved that essentially no changes occurred during the course of the study. This was expected because the whole buffer capacity of the blood system should contribute to the stabilisation of the pH.

For the preparation and stabilisation of the in vitro samples the aim was to maintain physiological pH. It turned out, however, that noticeable changes occurred during comparatively short time periods. This finding will be discussed later.

Performance of the Measurements

The hafnium labeled serum was placed in perspex tubes which were sealed. The samples were studied at room temperature. The recording time for a single measurement was between one and five days dependent on the activity of the probe. Since for the in vitro studies the sample material seemed to undergo changes with time the measurements were repeated at longer time intervals up to four months after preparation.

RESULTS

In Vivo Studies:

Five different samples have been studied. The details of the injected complexes (Hf-NTA, Hf-oxalate, or Hf-citrate), the incubation times (12 to 60 min), and the age of the samples after the serum has been collected (3 to 60 days) are listed in Table 2. Independent of these conditions three characteristically different spectrum components have been observed. They have been attributed to at least three chemically different binding configurations of the Hf ions. Two of these are considered as characteristic for specific hafnium binding to transferrin because of the small values of the frequency distribution width parameters δ_1 and δ_2. For the third fraction the frequency distribution was rather broad. Thus this component might be due to nonspecific binding of Hf ions in many different configurations.

The fractional contributions f_1 and f_2 to the spectra are given in Table 2. The third fraction f_3 amounted on the average to about 25%. The values of the characteristic parameters, i.e., quadrupole coupling constant ν_Q, asymmetry parameter η, and frequency distribution factor δ are listed in Table 4. Due to the large value of δ_3 the asymmetry parameter could only be determined for the fractions f_1 and f_2. The analysis of the data was thus performed assuming $\eta_3 = 0$.

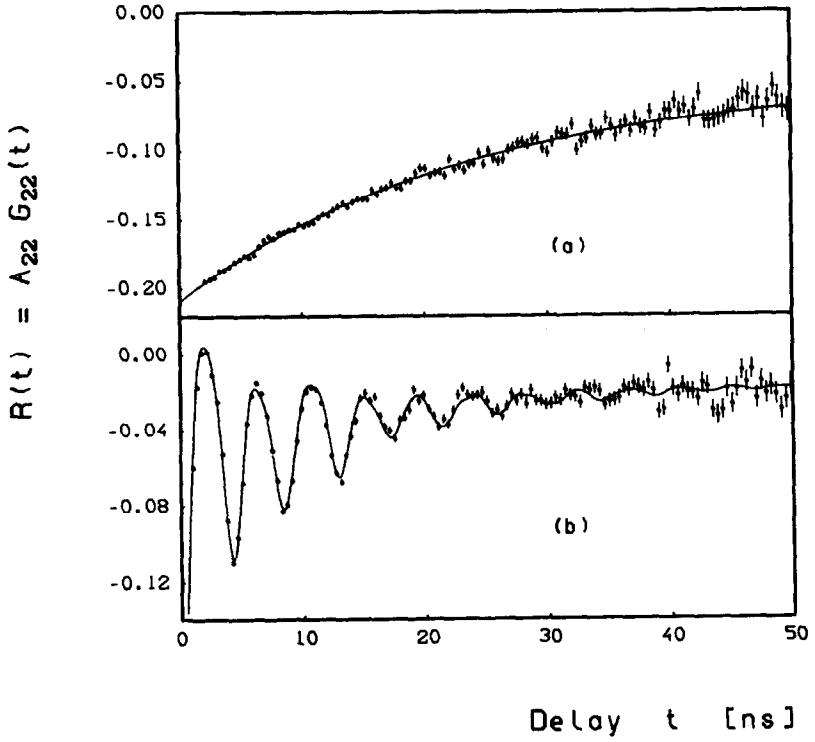
TABLE 2. Measurements on In Vivo ^{181}Hf-Labeled Rat Serum Transferrin

Sample no.	Injected complex	t_{inc} (min)	[Hf] (nmol)	[Hf]:[UIBC] (*)	Age of the sample (d)	f_1 (%)	f_2 (%)	Measurement no.
I	Hf-NTA	15	0.6	1:60	5	57±2	12±2	1
					14	63±5	6±4	2
II	Hf-NTA	15	0.8	1:45	7	51±2	21±4	3
					13	57±2	9±3	4
					25	61±2	6±2	5
					60	57±2	4±2	6
III	Hf-oxalate	15	1.3	1:28	4	43±1	20±2	7
					23	60±1	9±3	8
IV	Hf-citrate	60	3.0	1:12	3	39±1	28±2	9
					10	46±1	5±2	10
V	Hf-citrate	12	5.1	1:7.0	4	30±3	34±3	11

(*) The uncertainty of these ratios is approximately 15%.

For the samples prepared with Hf-NTA and Hf-oxalate fraction f_1 was dominant in all the spectra. Its percentage increased (and seemed to saturate) with the age of the sample. Figure 1 shows a typical spectrum taken with a rat serum sample prepared by Hf-NTA injection (measurement 5 in Table 2). The spectral response of the Hf-NTA solution is given for comparison.

FIGURE 1. Typical $R(t)$-spectrum of in vivo prepared rat serum transferrin (Fig. 1b). By comparison the spectrum of the ^{181}Hf-NTA injection solution (Fig. 1a) is due to fast relaxation characteristic for light hafnium complexes in liquid samples. (relaxation constant λ = 43 MHz).

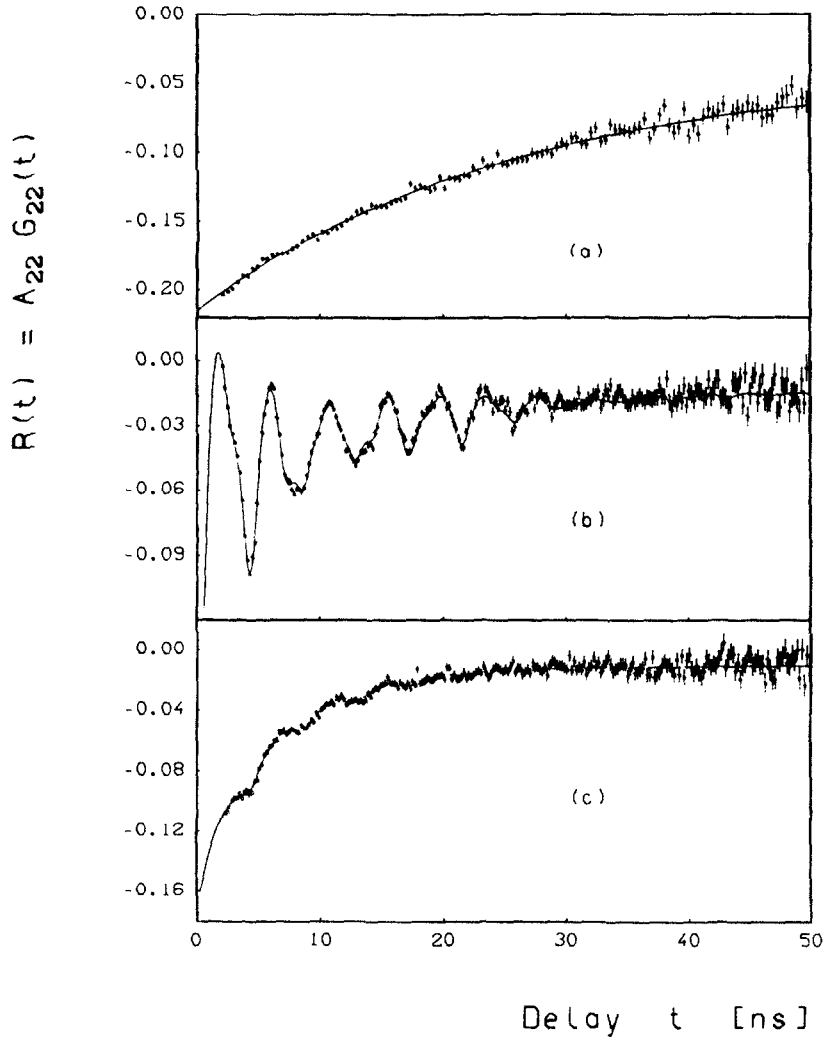


For the Hf-citrate samples the two fractions f_1 and f_2 were present in the spectra with nearly the same intensity. It should be mentioned that these samples were rather unstable and presented considerable changes after 1 or 2 weeks (see Fig. 2). After this period the TDPAC measurements showed a strong relaxation damping characteristic for low molecular Hf complexes. By gel chromatography it could indeed be demonstrated that only a small fraction of the Hf ions was still bound to transferrin.

In Vitro Results

In this series five samples with different [Hf]:[transferrin] ratios were prepared. In repeated measurements they were studied at time intervals up to 130 days (see Table

FIGURE 2. $R(t)$-spectra of ^{181}Hf-citrate samples. The injection solution (a) shows a fast relaxation spectrum (λ = 40 MHz). For a freshly prepared rat serum sample a well defined hafnium-transferrin spectrum (b) is obtained. The spectrum of the same sample, measured two weeks later (c) is dominated by a strong relaxation damping (λ = 162 MHz) typical for low-molecular hafnium complexes.



3). The hafnium concentrations were determined and limited, respectively, by the need for a ^{181}Hf activity of at least 5 μCi (corresponding to 2.3 nmol hafnium) in a maximal probe volume of about 1 cm^3 and a preponderant binding of the hafnium to transferrin. The latter condition was fulfilled up to about 11.5 nmol Hf/ml. These requirements resulted in ratios K of hafnium with respect to the UIBC of the transferrin molecule of between K = 1:15.6 and K = 1:3.1.

The characteristic part of the spectra could be analysed in complete analogy to the in vivo data, assuming three different fractions. The parameters are therefore listed together with the in vivo results in Table 4. For comparatively high hafnium concentrations, i.e., K = 1:3.7 and 1:3.1, the relaxation behaviour of the spectra pointed towards a fraction of hafnium bound to a low molecular component. In the evaluation procedure this fraction f_4 was taken into consideration by a fast relaxation term [24]:

$$G_{22}^{(4)}(t) = f_4 e^{-\lambda' t}.$$

For small amounts of hafnium, i.e., for concentration ratios K < 1:7.8 fraction 1 clearly predominates in the spectra. As can be seen from Table 3 the percentage of this fraction increases with time whereas the percentage that has to be assigned to fraction 2 decreases by equivalent amounts. After about 40 days a steady state seemed to be reached. Then only 10% of the Hf ions experience a quadrupole interaction that can be attributed to binding conditions for which the parameters of fraction 2 are characteristic.

In turn, for values of K > 1:5.2 the Hf ions prefer binding sites of the type corresponding to fraction 2. Again this preference is increased with time. The percentage that could be attributed to fraction 1 leveled out finally between 5% and 8%.

TABLE 3. Measurements on In Vitro ^{181}Hf-Labeled Rat Serum Transferrin

Sample no.	[Hf] (nmol)	[Hf]:[UIBC] (*)	Final pH value	Age of the sample (d)	f_1 (%)	f_2 (%)	Measurement no.
I	2.3	1:16	7.5	2	46±2	19±3	1
				16	57±2	11±3	2
II	4.6	1:7.8	7.8	5	47±2	19±3	3
				19	54±2	11±2	4
				42	56±2	10±2	5
III	6.9	1:5.2	9.2	7	43±2	17±3	6
				21	22±1	40±2	7
				38	7±1	57±2	8
IV	9.6	1:3.7	9.5	1	11±1	27±3	9
				6	4±1	49±3	10
				129	5±1	45±4	11
V	11.5	1:3.1	9.5	1	22±1	19±3	12
				2	11±1	19±4	13
				10	4±1	32±4	14
				128	8±1	19±3	15

(*) The uncertainty of these ratios is approximately 15%.

TABLE 4. Quadrupole-Coupling Parameters for ^{181}Hf Binding to Rat Serum Transferrin

		ν_Q (MHz)	η	δ (%)	$\lambda^{(*)}$ (MHz)	V_{zz} (10^{17} V/cm^2)	V_{yy} (10^{17} V/cm^2)	V_{xx} (10^{17} V/cm^2)
fraction	in vivo preparations	902 ± 4	0.92 ± 0.01	2.5 ± 0.3	62 ± 4			
f_1	in vitro preparations	900 ± 4	0.93 ± 0.01	2.4 ± 0.3	61 ± 1			
	mean values	901 ± 1	0.93 ± 0.01	2.5 ± 0.1	62 ± 2	15.8 ± 0.4	15.2 ± 0.4	0.55 ± 0.08
fraction	in vivo preparations	1463 ± 6	0.529 ± 0.003	0				
f_2	in vitro preparations	1473 ± 6	0.525 ± 0.002	0.4 ± 0.2				
	mean values	1468 ± 5	0.526 ± 0.002	0		25.7 ± 0.6	19.6 ± 0.4	6.1 ± 0.1
fraction f_3 (nonspecific binding)	mean values	939 ± 3	0	9.0 ± 0.2				

(*) The 3 fractions were fitted with the same relaxation constant.

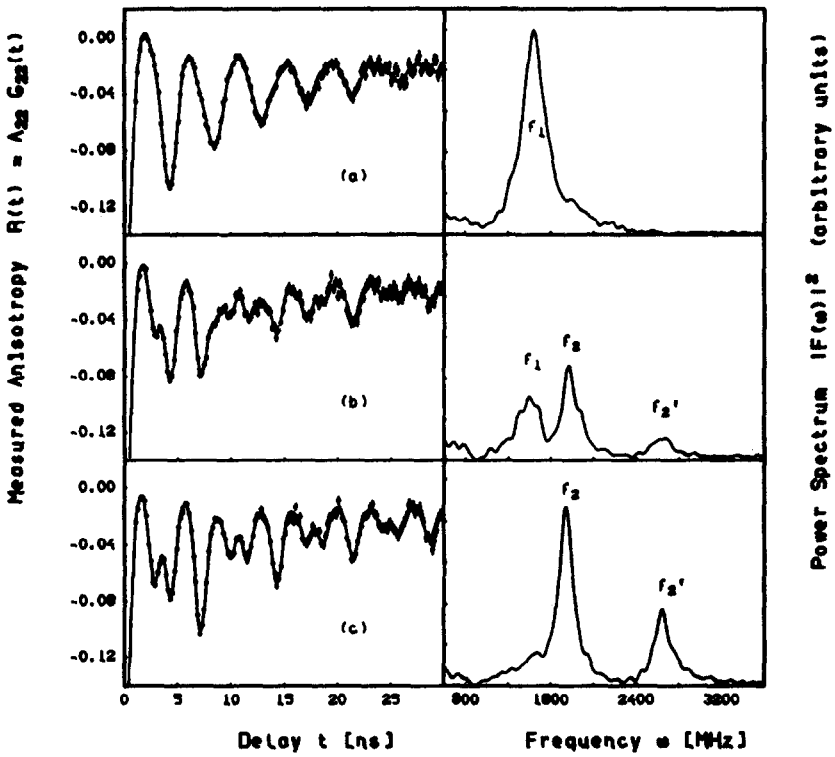


FIGURE 3. $R(t)$-spectra of in vitro prepared ^{181}Hf rat serum samples measured at different pH values between pH = 7.5 (a) and pH = 9.2 (c). The related Fourier transforms show the characteristic frequencies corresponding to the two specific binding configurations. For the second configuration the lowest two quadrupole frequencies (marked f_2 and f_2') are well separated. Because of the large asymmetry parameter of the first configuration these two frequencies are not resolved (line marked f_1).

In Figure 3, three characteristic spectra together with their relevant Fourier transforms are given. They correspond to measurements 5, 7, and 8, respectively, as quoted in Table 3.

It has been mentioned earlier that the in vitro prepared samples underwent a change of pH. With elapsing time the originally physiological pH rose to the final values which are listed in Table 3. It is obvious that the amount of change is clearly related to the hafnium concentration. The more hafnium that was used for preparing the solution the higher was the final pH. For samples with a predominant fraction 1 the final pH stayed below 8. If, however, fraction 2 was essentially observed then the final pH reached higher values, even >9. Thus a further relation exists between the relative distribution of Hf ions with respect to the two binding configurations and the observed final pH.

DISCUSSION

The two observed fractions f_1 and f_2 are attributed to two characteristic binding configurations for the Hf ions in transferrin. A comparison of the TDPAC parameters taken from the in vivo and the in vitro measurements shows that there are no

significant differences for either fraction. The average values of the parameters for both study series are listed in Table 4. Also listed are the calculated values for the electric field axial components $|V_{zz}|$, $|V_{yy}|$, and $|V_{xx}|$. The quadrupole moment of the relevant intermediate state in ^{181}Ta was taken as $Q = (2.36 \pm 0.05)$ barn [25].

In contrast to earlier TDPAC studies of indium transferrin [26, 27] the hyperfine interaction parameters have been determined here with great accuracy. This was possible because the $R(t)$-spectra of the ^{181}Hf measurements show nearly undamped oscillations with large amplitudes. These clean spectra are very probably due to the absence of serious after effects following the β-decay of ^{181}Hf in contrast to ^{111}In which decays by electron capture. It has been demonstrated that the modulation pattern in TDPAC measurements can be completely attenuated by a preceding electron capture in electrically nonconducting substances [28].

The field gradient distribution factors for the two specific fractions f_1 and f_2 are comparatively small. The observed attenuation of the $R(t)$-spectra is essentially due to fluctuating electric field gradients which lead to a characteristic relaxation damping. The relaxation can be parameterized by a correlation time τ_c. In cases of fast or slow relaxation (in comparison to the inverse value of the electric quadrupole frequency) the correlation time can be evaluated directly from the measured relaxation constant [29]:

$$\tau_c = 1/\lambda, \qquad \text{for slow relaxation and}$$

$$\tau_c = 400\lambda/(100.8\pi^2\nu_Q^2(1+\eta^2/3)), \qquad \text{for fast relaxation.}$$

In liquid samples large molecules such as transferrin move rather slowly. These Brownian rotational motions lead therefore to fluctuations of the electric field gradients which manifest themselves by a slow relaxation behaviour. The rotational correlation time may be estimated for a spherical molecule according to the Debye model [30]:

$$\tau_c = V\tilde{\eta}/kT.$$

With the viscosity of the serum $\tilde{\eta} = 1.7 \times 10^{-3}$ kg/ms [31] and the molecular volume of transferrin $V = 169 \times 10^{-27}$ m^3 [32] for $T = 295$ K the characteristic correlation time would be $\tau_c = 71$ ns. This estimation has to be compared to the measured relaxation constant (see Table 4) which corresponds to a correlation time $\tau_c = 16 \pm 1$ ns. For the general case of an asymmetric molecule the mean correlation time is due to 3 rotational degrees of freedom. Its value would be even higher than the result of the Debye approximation for spherical molecules [33]. Obviously the correlation time expected for reoriental motion of the whole transferrin molecule is significantly larger than the value observed in the measurements reported here. Internal reorientations within the protein molecule or effects due to fluid dynamics have to be assumed to explain the experimental results. Temperature dependent measurements will be necessary for a better understanding of these relaxation phenomena. Such systematic investigations are in progress and will be discussed in a separate publication [34].

The sample material has been taken from normal rat serum. An additional series of measurements was carried out where the samples were prepared starting from human apotransferrin. TDPAC measurements with these iron-free ^{181}Hf transferrin samples (see Fig. 4) showed the same specific Hf-binding configurations as the rat serum data. The existence of equivalent and well defined binding states in rat serum and in human apotransferrin indicates clearly that specific hafnium binding to transferrin was observed. It further confirms that the incubation medium has no influence on the

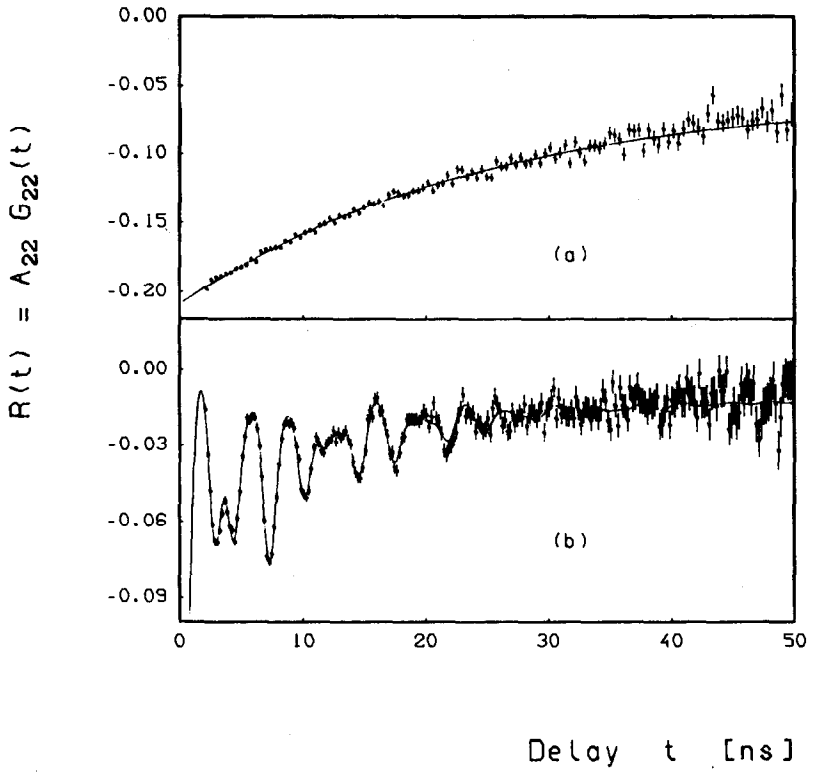


FIGURE 4. $R(t)$-spectrum of *in vitro* prepared human apotransferrin (Fig. 4b). A control measurement performed with the incubation medium alone (Fig. 4a) shows a fast relaxation behavior typical for the low-molecular Hf–NTA complex.

chemical nature of the binding ligands. The quadrupole parameters for rat and human serum transferrin turned out to be slightly different, suggesting a small species-dependent differentiation of the metal-binding sites [34].

For a discussion of the occupation numbers for both sites one has to take into consideration that about 51% of the binding capacity of the transferrin molecules in rat serum are already taken by iron. For rat serum a random distribution of iron between the C- and N-terminal sites has been postulated [35]. Most recently for freshly drawn human serum a ratio Fe_N:Fe_C of 1.7:1 was reported [4]. Independent of the exact value of the ratio Fe_N:Fe_C it seems reasonable to assume that sufficient free binding capacity should remain in either terminal site even for the largest hafnium concentrations used here.

The starting chelates were prepared from hafnium chloride solutions. Therefore increasing hafnium concentrations were accompanied by higher salt concentrations. It has been shown that such higher salt concentrations lead to a preferred binding to the N-terminal site [36]. It is still not understood which mechanism caused the rise of the pH value up to >9 for the samples with higher hafnium concentrations. However it is well known that high pH values favor the metal binding to the N-terminal site in transferrin [37]. Comparing these observations with the results of the TDPAC measurements presented here it seems reasonable to identify fraction 2 with Hf ions

bound to the N-terminal site. Further it was observed that the population of this site requires a somewhat longer incubation time. This result is consistent with the findings of van Eijk et al. [38, 39] for the population of the N-terminal site when Fe–NTA is used (site B = N-terminal site and site A = C-terminal site in the papers cited). The same authors claim a preponderant population of the C-terminal site for pH < 8.3. If a similar behavior of Hf and Fe ions is assumed then fraction 1 and fraction 2 of the TDPAC-measurements may be attributed to ions populating the C- and the N-terminal sites of transferrin, respectively. These studies then should be considered as a clear demonstration of the chemical nonequivalence of the two metal-binding sites in transferrin and would imply that Hf ions at low pH bound to the C-terminal site migrate to the N-terminal site as the pH increases.

It should be mentioned, however, that some authors postulate configurational changes and subsequently a change of the site character as a consequence of the formation of a new conformational substate at high pH values [37, 40–43]. Whether such a conformational change should affect the character of only one or both sites remains open. Thus this hypothesis still permits the assumption that both sites are chemically equivalent and only alter their configuration due to a conformational change of the molecule which, in turn, results from the influence of pH, salt concentration, temperature and possibly other parameters. In this framework two possible interpretations of our data have to be considered. Either at low pH hafnium is bound preferentially to one site and this site is subject to a configurational change with rising pH that makes it identical with the second one (as previously suggested, see e.g., [40]), or both binding sites are chemically identical and therefore equally populated and change their configuration in the same way with increasing pH.

A further comment concerns the symmetry of the binding configuration of fraction 2. The observed value $\eta = 0.526$ for the asymmetry coefficient is typical for a series of hafnium compounds such as Hf–sulfate [44], Hf–tropolonate [45], various Hf–oxalate complexes [46], and Hf–oxychloride [47]. It is well known that in all these compounds the Hf ions are arranged in a dodecahedral environment (D_{2d}) and coordinated by eight oxygen atoms. Whether this symmetry arrangement is also true for hafnium in transferrin remains to be seen.

Apart from the two fractions observed for Hf ions in transferrin which have been attributed to specific binding conditions a third fraction appeared in the data of all measurements (see Table 4). Its relative contribution was independent of the special sample condition and amounted to about 25% to 30%. It might be considered as resulting from nonspecifically bound Hf ions, i.e., ions that experience quadrupole interactions due to many different binding configurations. Another explanation for this fraction of Hf ions could lie with physicochemical effects in connection with the radioactive decay of the probe nucleus. These effects have been observed for many organic hafnium-compounds (see, e.g., [29]). The observed gamma–gamma cascade in ^{181}Ta is preceded by the β-decay of ^{181}Hf. The recoil energy of 1.6 eV may excite parts of the transferrin molecule into metastable states with slightly different chemical configurations. Electron shake-off processes after the β-decay may lead to a strongly disturbed field gradient at the binding site. This could mean that even if all Hf ions are specifically bound to transferrin the well-defined environment is perturbed for a relevant percentage of the ^{181}Hf probe atoms.

We are greatly indebted to Professor P. Aisen for critically reading the manuscript and for many substantial and helpful comments.

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Received February 4, 1986, Accepted April 29, 1986.