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Perturbed angular correlation studies of the metal-binding sites in ovotransferrin and its C- and N-terminal halves

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The perturbed angular correlation (PAC) technique has been applied to study the electric quadrupole interaction of ^{181}Hf nuclei at the binding sites of ovotransferrin (OTF) molecules. Two specific electric field gradients were observed. Their relative intensities depend on the pH value and the temperature of the samples, whereas the electric quadrupole interaction parameters themselves remain unaffected. In order to compare the binding sites in OTF, experiments with N- and C-terminal half-molecules were performed. Both specific configurations are observed at the N-terminal and at the C-terminal binding site with similar quadrupole parameters as for the intact protein. Remarkably, the stability of the hafnium binding to the C-terminal fragment appears to be reduced as compared with the N-terminal half and the intact protein.

Keywords: electric quadrupole interaction, ^{181}Hf, ovotransferrin, perturbed angular correlations, relaxation

Introduction

The transferrins are a homologous group of glycoproteins with the property of reversibly binding iron and a series of other metal ions together with a carbonate anion. The latter is called the synergistic anion. Apart from serum transferrin (TF) and lactoferrin (LF), ovotransferrin (OTF) is one of the well-characterized members of the transferrin family. The high degree of similarity of the primary and tertiary structure establishes the close relationship of the three proteins. They consist of a single chain of approximately 700 amino acids with a molecular mass of approximately 80 kDa. Crystallographic X-ray structure determinations of LF by Anderson *et al.* (1989, 1990) and Baker *et al.* (1991), and of TF by Bailey *et al.* (1988) have shown that these proteins are folded into two structurally similar regions, the N- and the C-terminal lobe, with an internal sequence homology (identical residues in corresponding positions) for human TF and hen OTF of 41 and 33%, respectively (Metz-Boutigue *et al.* 1984). A specific binding site with high affinity for metabolic iron and other metals is located in each of the two lobes. The main function of TF is the transport of iron from the sites of uptake to the sites of storage and utilization. The transferrins also have a bacteriocidal function: they deprive micro-organisms of iron which is essential for bacterial growth. For recent reviews on the transferrins see, for example, Brock (1985), Aisen & Harris (1989) and de Jong *et al.* (1990). Apart from iron, many other metals bind to the transferrins. Representative of the binding of non-physiological metals, hafnium is used for the investigations in the present study and in past work with TF (Then *et al.* 1983).

For our studies of the metal binding to the specific sites of OTF we applied the nuclear spectroscopic perturbed angular correlation (PAC) technique. This method allows detection of the electromagnetic interaction between the probe nucleus and the local electronic structure. The interaction is sensitive to the geometry, the coordination number and the type of ligands. The specific metal binding and the symmetry of the ligand field arrangement can be

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inferred from the electric field gradient (EFG) observed at the position of the probe nuclei. The sensitivity of the electric nuclear quadrupole interaction (NQI) decreases with $1/r^3$ and therefore only the close surroundings of the probe nucleus contribute to the signal. This high sensitivity of the PAC signal to the first coordination sphere allows a detailed study of the ligand fields and their dependence on external conditions like pH and temperature. The main advantage of the PAC technique is that, in contrast to many other methods, the experiments can be performed under nearly physiological conditions: liquid samples with low metal and protein concentrations in the order of nmol/ml can be studied. An excellent probe nucleus for the PAC technique is the isotope ^{181}Hf because detailed information can be obtained from the hafnium spectra, as will be shown in this paper.

Materials and Methods

The PAC technique

The PAC technique is based on the observation of the time dependent angular correlation of two successive γ-rays emitted by the probe nuclei. The NQI between the electric quadrupole moment Q of the probe nucleus and the EFG caused by the charge density distribution around the probe nucleus induces a time dependent modulation $G_{22}(t)$ of the correlation function. For the probe nucleus ^{181}Hf in polycrystalline or liquid material, the PAC signal $R(t)$ is given by

$$R(t) = A_{22}G_{22}(t)$$

where A_{22} is the anisotropy coefficient. For a detailed description of the method see Frauenfelder & Steffen (1965).

Due to the fact that the experiments with OTF samples were performed in the liquid phase, a time-dependent quadrupole interaction was expected as a result of random fluctuations in the perturbing fields. If the characteristic correlation time τ_c describing the fluctuations is large compared with $1/\omega_0$ then the following perturbation function G_{22} has to be used (Boyer & Baudry 1984)

$$G_{22}(t) = e^{-\lambda t} \sum_{k=0}^{3} s_{2k}(\eta) \cos(\omega_k t) e^{-\omega_k \delta t} \quad \text{with} \quad \lambda = \frac{1}{\tau_c}$$

where the $\omega_k = n_k(\eta)\omega_0$ are the observed angular frequencies. The amplitudes s_{2k} and the frequency ratios n_k depend on the asymmetry coefficient η which reveals deviations of the ligand field arrangement from axial symmetry. It is defined as

$$\eta = \frac{V_{xx} - V_{yy}}{V_{zz}} \quad \text{with} \quad 0 \leq \eta \leq 1$$

A Lorentzian distribution of the EFGs was assumed (Baudry *et al.* 1983). The parameter δ describes the width of this distribution. The Lorentzian shape results in a slightly better description of the data as compared with a Gaussain distribution. For spin $I = 5/2$ the angular frequency ω_0 is correlated to the quadrupole coupling constant ν_Q and the z-component of the EFG tensor **V** via the relation:

$$\nu_Q = \frac{10}{3\pi}\omega_0 = \frac{eQV_{zz}}{h}$$

where e is the unit charge, Q the nuclear quadrupole moment and h Planck's constant. The frequency ν_Q reflects the characteristic EFG at the sites of the hafnium ions.

In liquid samples with macromolecules of high molecular weight such as OTF the rotational movement is rather slow and the correlation time consequently long. Therefore a slow relaxation behavior was expected for the measurements with liquid OTF samples.

If more than one binding site exists for the probe ions, a superposition of the individual perturbation functions G_{22}^i has to be taken into account for describing the spectra. The amplitudes f_i correspond to the fraction of nuclei that experience a specific EFG.

However, in the case of rapid fluctuations, i.e. when the correlation time τ_c is small compared with $1/\omega_0$, the perturbation function is given by (Boyer & Baudry 1984)

$$G_{22}(t) = e^{-\lambda t} \quad \text{with} \quad \lambda \propto \tau_c$$

This will be the case for complexes of low molecular weight.

Data analysis

Eight coincidence spectra $N(\Theta, t)$ were recorded simultaneously with a conventional four-detector PAC spectrometer. Details of the set-up are described elsewhere (see, e.g. Heidinger *et al.* 1987). The PAC signal $R(t)$ is obtained from the coincidence spectra $N(\Theta, t)$ by forming the ratio

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

The calculated value for the effective anisotropy $A_{22}^{\text{eff}} = -0.23$ was verified experimentally. The NQI parameters were determined from the $R(t)$ spectra using a least-squares fit algorithm. For a first approximation of the asymmetry parameter and the quadrupole frequency it was helpful to calculate the Fourier-transformed spectra where the angular frequencies and their ratios can be easily extracted.

Sample preparation

The apo-form of hen OTF was purchased from Serva (Heidelberg, Germany). The C- and N-terminal half-molecules were prepared as described by Oe *et al.* (1988) except that the two lobes were quantitatively separated by electrofusing on an LKB electrofocusing column using 0.4% pH 4–8 (LKB) ampholytes. Following exhaustive

dialysis and concentration on an Amicon ultrafiltration apparatus, the samples were run over a Sephadex G-75 column (2.5 × 90 cm) to remove any residual ampholytes and sucrose. Purity was accessed by spectra ratios and SDS–PAGE. For pH values up to 8.0 the protein material was dissolved in 50 mM MES/50 mM HEPPS buffer with a phosphate content of 1–8 mM. This covers the range of the physiological phosphate concentrations. At higher pH values 100 mM Tris buffer was used. The protein concentration was 25 nmol ml^{-1} in a total sample volume of 0.5 ml.

Hafnium chloride solution with a specific activity ranging from 700 to 1500 MBq mg^{-1} ^{181}Hf in 2 M HCl was purchased from Amersham-Buchler (Braunschweig, Germany). The hafnium ions were complexed with a 4-fold excess of nitrilotriacetate (NTA) and the solution was then titrated with NaOH to just below the required pH. The pH was adjusted to the final value by adding small amounts of sodium bicarbonate. As shown by Then *et al.* (1983), hafnium then forms soluble complexes of Hf–NTA. After an incubation time of 15 min the ^{181}Hf–NTA solution (2.5 nmol Hf, 10 nmol NTA) was added to the protein solution for metal loading. Difference UV spectroscopy was used to establish that the hafnium ions were specifically bound to OTF.

Performance of the measurements

The OTF samples were sterilized by micro-filtration and placed in thin-walled, cylindrical perspex tubes. To avoid changes of the pH value due to changes of the CO_2 content the samples were sealed hermetically. Before the PAC measurements were started an incubation time of about 12 h was allowed. The experiments were performed at different temperatures between 270 and 320 K. The temperature of the samples was stabilized and checked using a Lauda compact thermostat (Lauda Königshofen, Germany) with an accuracy of better than 0.2 K. The recording time for a single spectrum was between 24 and 100 h, depending on the activity of the samples. The measurements were repeated at longer time intervals up to 12 weeks after the preparation.

Results

The subject of our studies is the metal binding sites characterized by the relevant EFGs and the influence of the sample preparation conditions on the metal binding. In previous studies it has been shown that the pH and the temperature influence the stability constant of the iron–transferrin complex (Chasteen & Williams 1981) and the protein dynamics (Schwab *et al.* 1992). Therefore the effect of these parameters on the metal binding sites was studied in the present experiments. At physiological pH values of about 7.5, PAC spectra (see Figure 1) were obtained that are characteristic for a single, sharp EFG of high asymmetry ($\eta \approx 0.9$). The corresponding binding configuration will be termed the low-pH configuration f_1. With increasing pH values a second, stronger EFG with a lower asymmetry parameter ($\eta \approx 0.6$) takes over. This high-pH configuration f_2 dominates the PAC signal above pH 9.0. As reported earlier for human serum transferrin (Taylor *et al.* 1988), the presence of phosphate is essential for the formation of f_1. This is also the case for OTF. The phosphate concentration required to give an intensive PAC signal for f_1 is in the order of the physiological values of 1–2 mM. Both EFGs for f_1 and f_2 are described by comparatively small values of the frequency distribution width of approximately 2%, which marks a well-defined binding state of the metal.

The influence of pH on the spectra and EFGs is shown in Figure 1. The characteristic low-pH configuration f_1 is observed for physiological pH 7.5, whereas for pH values as high as 9.5 only the high-pH configuration f_2 contributes to the spectrum. A superposition of both configurations with varying amplitude ratios is measured in a pH range between 7.5 and 9.5.

The Fourier spectra in Figure 1 show pronounced and well-resolved angular frequencies. The first frequency peak of the low-pH configuration f_1 seems to be somewhat broader than for f_2. This is, however, only due to the corresponding asymmetry parameter being approximately 0.9. Thus the first two frequencies are nearly identical. The frequency distribution in the Fourier spectra around $\omega \approx 0$ originates from the exponential damping due to the relaxation phenomena.

The optimum least-squares fit of the model to the data, however, was achieved when an additional third fraction f_3 was introduced. In contrast to f_1 and f_2 the frequency distribution width for f_3 turned out to be comparatively broad ($\delta \approx 16\%$), indicating a binding of the corresponding hafnium ions in several slightly different configurations. The amplitude for this third fraction was rather small (between 10 and 20%). Therefore no pronounced frequency peak can be seen in the Fourier spectra in Figure 1. For comparatively low pH values (pH < 7), however, fraction f_3 was dominant. The asymmetry parameter was assumed to be zero for this fraction f_3. Values above $\eta \approx 0.2$ resulted in a slightly higher sum of squares of deviations. The lowest angular frequency of f_3 is expected in the Fourier spectra in Figure 1 at about 900 Mrad s^{-1}. Due to parameter correlations the relaxation constant for f_3 could not be determined independent of δ. Therefore it was assumed to be identical to the relaxation constant for f_1 or f_2.

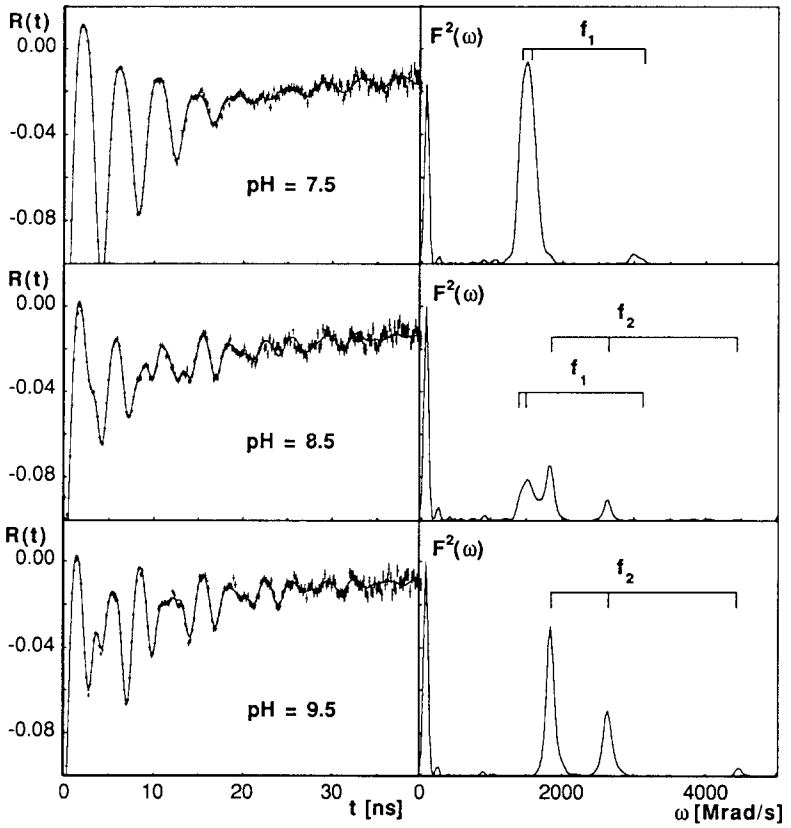

Figure 1. Typical $R(t)$ spectra and Fourier amplitudes (in arbitrary units) of ^{181}Hf–OTF taken with samples at pH 7.5, 8.5 and 9.5.

In Table 1 the complete set of NQI parameters is listed for the three fractions observed in OTF. Both fractions f_1 and f_2 must be attributed to clearly different, well-defined binding configurations of the hafnium ions. The frequencies as well as the asymmetry parameters of fraction f_1 and f_2 are significantly different, proving that the electron density distribution around the probe nucleus clearly changes when the pH value is altered. The relaxation constant is about 10 MHz higher for f_2, indicating that the dynamic processes are slighty different for the two binding configurations.

For the interpretation of the spectra it is necessary to determine where the binding sites of the probe metals are located. The existence of binding sites that are not identical to the specific metal sites has been shown, e.g. by Hutchens & Yip (1991). The essential criterion for the specific binding of metals is the results of competitive binding experiments (Aisen 1980). These are based on the fact that the transferrins are optimized for the binding of iron. The binding strength of other metals to the specific sites is smaller by some orders of magnitude than that for iron. Therefore iron will replace the other metals at the specific sites.

The specific binding of hafnium to OTF was verified in the following experiment. A Hf–OTF sample was measured before and after a

Table 1. NQI parameters[a] for ^{181}Hf–OTF measured at room temperature (about 300 K)

	ν_Q(MHz)	η	δ(%)	λ(MHz)
f_1	928.0 ± 1.5	0.886 ± 0.002	2.1 ± 0.1	59.0 ± 1.9
f_2	1473.1 ± 1.5	0.588 ± 0.001	1.6 ± 0.2	68.2 ± 1.9
f_3	953.6 ± 5.7	0	15.8 ± 1.1	as for f_1 or f_2

[a]The values given here are weighted means ± standard errors.

Fe(III)–NTA solution was added. The mole ratio of iron to OTF was 2:1, corresponding to one iron ion per binding site. The pH of the samples was adjusted to a value of 9.2. The results of the PAC measurements are shown in Figure 2. Before iron was added only the high-pH configuration could be detected for both temperatures.

After iron loading, single exponential decay spectra were observed. The relaxation constant decreases with increasing temperature thus proving that the hafnium nuclei undergo fast relaxation. The signal amplitudes were identical within the parameter errors. The experiment demonstrates that the hafnium ions were originally located at the specific binding sites of OTF. They are removed from these sites when iron is added to the solution. The hafnium ions then form low-molecular species and undergo fast relaxation. It is plausible to assume the formation of Hf–NTA complexes because the relaxation constants observed here are in good agreement with the values measured previously (Then *et al.* 1983).

A further essential condition to assure that the data are significant is proof of the stability of the binding of hafnium to the OTF molecules, i.e. no changes of the samples take place over time. Therefore the PAC signals were recorded in two time regimes: shortly after the sample preparation so as to take into account possible incubation effects and at longer time periods of several weeks for the test of the long-term stability. The corresponding amplitudes are plotted in Figure 3. A semi-logarithmic representation of the time axis was chosen so that it was possible to resolve both short and long time scales. The characteristic amplitude f_2 reaches its final value approximately 10 h after the sample preparation. At very short times up to 6 h a new fraction f_r dominates which showed a fast relaxation behavior. The relaxation constant at 300 K was 36 MHz, corresponding to the value

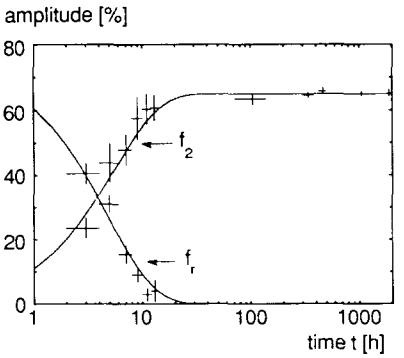

Figure 3. Amplitudes of the specific fraction f_2 and of the fast relaxation fraction f_r as a function of time for a Hf–OTF sample prepared at pH 9.2. The horizontal bars give the duration of the experiment, the vertical bars represent the errors of the amplitudes. Exponential functions were fitted to the data to guide the eye.

previously observed for Hf–NTA molecules in solution (Then *et al.* 1983). After 12 h no further changes in the spectra can be observed and the long-term stability extends over a period of several weeks.

It is well-known that the dynamics and the function of proteins are influenced by temperature. To investigate the changes at the metal sites in OTF a series of experiments were performed in a temperature range between 260 and 320 K. The temperature 268 K marks the freezing point of the samples. Below this temperature, where the phase transition takes place, the EFGs were smeared out and the parameters could not be determined accurately. Above 320 K an irreversible reduction of the PAC signal amplitude was observed: above this critical temperature the hafnium ions are released from the protein.

The frequency, the frequency distribution width and the asymmetry parameter show only very slight

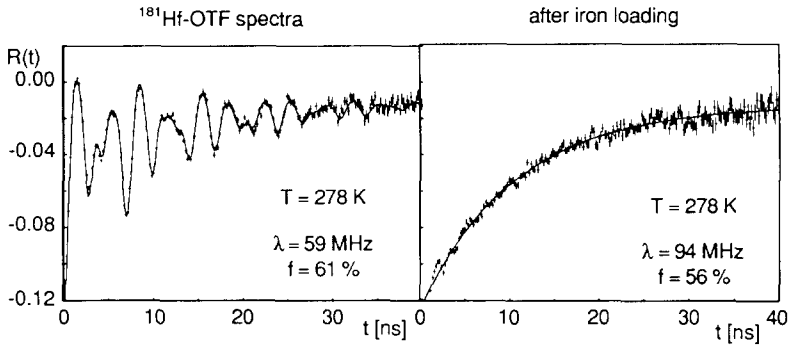

Figure 2. PAC spectra of ^{181}Hf–OTF samples before and after adding Fe(III)–NTA. After iron loading fast relaxation was observed.

variations with temperature for both configurations. The changes of these parameters of less than 1% are of the order of the parameter errors. Consequently temperature does not have a marked influence on the EFGs and no direct impact on the ligand field arrangement around the metal site is observed. The amplitudes of f_1 and f_2, however, were shown to be strongly influenced by the temperature. Figure 4 shows the relative intensities of the observed fractions f_1 and f_2 taken as a function of temperature. Raising the temperature clearly favors fraction f_2. The less well-defined fraction f_3 as well as the sum of all amplitudes remain almost constant over the whole temperature range.

An essential parameter that is also influenced by the temperature is the relaxation constant. A slow relaxation behavior has been established previously and it can be shown that it is composed of internal dynamics as well as of reorientation of the whole molecule (Schwab *et al.* 1992).

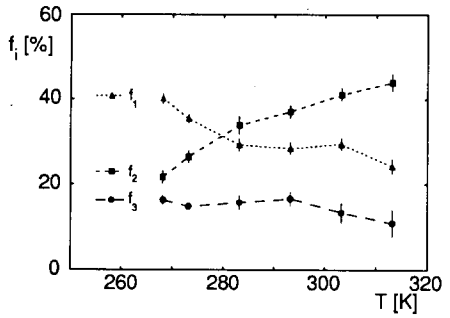

Figure 4. Temperature dependence of fractions f_1, f_2 and f_3 for an OTF sample prepared at pH 9.2 with 8mM phosphate.

The question arises whether the specific configurations can be attributed to one of the two binding sites in the protein. Altering the ratio of the two binding types by varying, for example, the pH value of the solution or the temperature, then necessarily would imply a migration of metal ions from one binding site to the other as first postulated by van Eijk *et al.* (1978). Alternatively it can be assumed that both binding sites are capable of forming the two characteristics and different binding configurations by alteration of the specific ligand arrangement at the metal site and/or conformational changes. To address these questions, additional information was sought from experiments with C- and N-terminal half-molecule fragments containing intact binding sites.

The binding of the hafnium ions to the two half-molecule fragments was established using difference UV spectroscopy (data not shown). As with OTF the PAC experiments were performed under the same controlled conditions. Typical spectra taken at low and high pH for the two half-molecules of OTF are shown in Figure 5.

Two different binding states were observed with an amplitude ratio f_1/f_2 dependent on the pH value of the samples. Apart from a stronger damping that can be seen clearly in the spectra, the structures in the $R(t)$ spectra for the intact protein are reproduced. A quantitative comparison of NQI para-

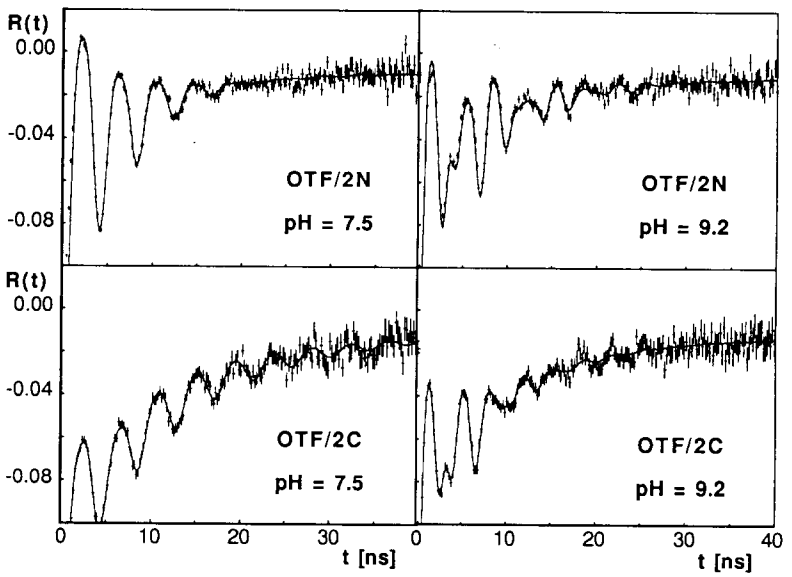

Figure 5. PAC Spectra of ^{181}Hf-labeled C- and N-terminal OTF half-molecule fragments at pH 7.5 and 9.2. For the C-terminal fragment an additional, rapidly relaxing fraction had to be taken into account.

meters of the two OTF half-molecules is given in Table 2. The third, less well-defined fraction f_3 was also observed with both fragments. Although the NQI parameters for f_3 could not be determined with the same accuracy as for the intact protein they were found to be close to the former values.

A comparison of the N-terminal half (OTF/2N) with the intact molecule shows excellent agreement of the NQI parameters with the exception of the relaxation constant. The relaxation constant was expected to be higher because the reduced volume for the protein halves leads to a smaller correlation time. Slight deviations from the parameters for the intact protein were found for the C-terminal fragment (OTF/2C). The frequencies and asymmetry parameters are some percent off the values for OTF. Nevertheless, it is evident from these results that each fragment is capable of forming both the low-pH and the high-pH configuration.

In addition to the quasi-static interaction model for the C-terminal fragment it is necessary to take into account a further fraction f_4 that is best described by an exponential function (fast relaxation). The corresponding amplitude has been shown to be time dependent, i.e. 200 h after the sample preparation, the specific PAC signal has vanished in favor of the exponential signal. The PAC amplitudes of both molecular halves are shown as a function of time in Figure 6. This instability is only observed with the C-terminal half, whereas the N-terminal half-molecule of OTF showed no changes for weeks.

For the determination of the relaxation regime for fraction f_4 and of the correlation time the relaxation constant was taken as a function of temperature. The results of the experiments are given in Figure 7.

For relaxation processes with a constant activation energy E_a, the transition rate W or the inverse correlation time is given by an Arrhenius relation (Shinar *et al*. 1984):

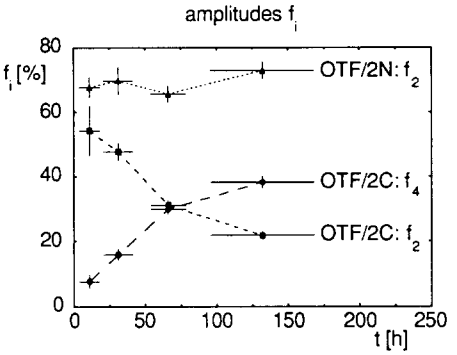

Figure 6. Amplitudes of the PAC signals for OTF/2C and OTF/2N at pH 9.2 as a function of the time after preparation of the samples. The horizontal bars give the duration of the experiment, the vertical bars represent the errors of the amplitudes. The meaurements were started 10 h after the sample preparation. The fraction f_4 corresponds to fast relaxation.

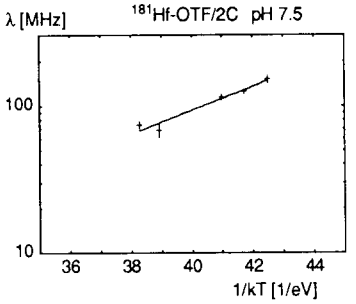

Figure 7. Relaxation constant for f_4 of ^{181}Hf loaded OTF/2C. An activation energy $E_a = 0.18$ eV was deduced for this fraction.

$$W = \frac{1}{\tau_c} = W_0 \exp\left(\frac{-E_a}{k_B T}\right)$$

where k_B is the Boltzmann constant. The parameter λ for fraction f_4, shown in Figure 7, is characterized

Table 2. NQI parameters[a] for ^{181}Hf–OTF/2N and ^{181}Hf–OTF/2C taken at about 300 K

	ν_Q(MHz)	η	δ(%)	λ(MHz)
OTF/2N				
f_1	931.7 ± 1.7	0.877 ± 0.005	2.2 ± 0.1	102.6 ± 10.3
f_2	1479.5 ± 2.6	0.587 ± 0.001	2.1 ± 0.3	92.1 ± 6.6
OTF/2C				
f_1	901.8 ± 2.2	0.952 ± 0.082	2–5[b]	100–150[b]
f_2	1561.9 ± 2.9	0.524 ± 0.004	2–5[b]	100–150[b]

[a]The values given here are weighted means ± standard errors.
[b]Due to parameters correlations δ and λ could not be determined independently. The results of the fit were always found in the range given here.

by fast relaxation, as can be deduced from the positive slope. This behavior is usually observed for relatively small molecules of less than about 10 kDa. Fraction f_4 has to be interpreted as related to probe nuclei that are bound in low molecular weight complexes. Taking into consideration the time-dependence of the amplitudes shown in Figure 6, it becomes obvious that within 200 h the hafnium ions are released from the C-terminal half-molecule and form complexes of relatively low weight. The values of the relaxation constant for f_4 compare well with the values derived for Hf–NTA (Then *et al.* 1983). The activation energies (0.18 eV for f_4 and 0.13 eV for NTA) derived from the Arrhenius plots are also rather similar and therefore it may be speculated that the Hf–NTA species is formed again.

Discussion

By using the PAC method with liquid samples of hafnium labeled OTF, information about the electronic environment of the probe nuclei has been obtained. Two well-defined field gradients with small distribution widths have been observed in the experiments. They represent the characteristic binding configurations of the probe metal hafnium in OTF. The unusually small distribution widths δ indicate that the specific binding of hafnium to OTF is observed. The relative intensities of the two fractions f_1 and f_2, in contrast to the NQI parameters, are influenced by the pH value of the samples and the temperature. The difference of the fractions f_1 and f_2 as to asymmetry and strength is comparatively large. Since the sensitivity of the PAC technique decreases with $1/r^3$, this difference cannot be explained by changes of the protein structure at a distance of more than 5 Å from the probe nuclei. Alterations of the first coordination sphere around the probe metal, e.g. due to an exchange of one of the ligands, are more plausible. Also the fact that different relaxation constants and therefore different dynamics were observed for the two binding configurations contribute to this hypothesis. These findings fit well into the picture given by other authors: a change of the site character as a consequence of the formation of new configurational substates at higher pH values has been postulated, e.g. by Chasteen & Williams (1981). It has also been speculated that the release of iron requires a conformational change from a closed to an open molecular conformation (Baker *et al.* 1991). This conformational change may be affected or induced by changes of the pH value and the temperature. Similar influences of these parameters on the spectra are found in our PAC experiments. The specific binding of hafnium is accomplished within 12 h, a relatively long time as compared with the binding of iron, where time ranges of seconds up to minutes are reported (Taniguchi *et al.* 1990).

For an optimum fit to the data it was necessary to introduce a third fraction. Taking into consideration the relatively broad frequency distribution, it cannot be excluded that several different binding configurations are observed, which are experimentally unresolved. Since this fraction is also best described by the slow relaxation model, it has to be concluded that the nuclei responsible for f_3 are still liganded to OTF. This fraction might be considered as originating from hafnium nuclei that are not located at the specific metal binding sites but are non-specifically bound to the protein. A further explanation could lie in the β^- decay preceding the γ–γ cascade that is observed for the experiments. The recoil energy of the hafnium nucleus (1.6 eV) is too low to break up the covalent bonds and to release the metal, but it may transfer energy to the OTF molecule and excite it into metastable states or induce small conformational changes, thus disturbing the electronic environment of the probe nuclei. The observation that fraction f_3 is not temperature dependent especially supports this interpretation.

It is interesting to compare the results for the intact protein molecule OTF with the results for the N- and C-terminal half-molecules. Again two specific binding configurations were derived for both halves. In terms of the NQI parameters the following results have been deduced. The NQI parameters ν_Q and η, characterizing the ligand field arrangement, are exactly the same for the N-terminal fragment as for the intact protein. In contrast, the metal binding configuration in the C-terminal half-molecule is slightly different. The NQI parameters ν_Q and η deviate from the values for the N-terminal fragment, indicating a small difference in the ligand arrangement. Furthermore a fraction f_4 of fast relaxation had to be introduced to describe the $R(t)$ spectra of OTF/2C. Remarkably, the ability of the C-terminal fragment to bind hafnium in both characteristic configurations appears to be reduced as compared with the N-terminal fragment. The hafnium coordination in the N-terminal fragment shows considerable stability. The proportion of rapidly relaxing fraction f_4, observed in the PAC spectra for the C-terminal fragment, increased with time, demonstrating that the specific hafnium binding configuration in this fragment is less stable than in the N-terminal fragment. The larger relaxation constant for both fragments as compared with the

intact protein must be ascribed to the smaller volume leading to a faster reorientation of the molecules.

The fast relaxation observed in the intact protein at very short times after the sample preparation as well as in the unstable C-terminal half-molecule may be attributed to low molecular weight complexes. From the measured relaxation constant and from former experiments with aqueous solutions of Hf–NTA, it is inferred that the formation of Hf–NTA complexes is being observed. On the other hand, hafnium hydroxides can be excluded because with the formation of insoluble complexes, a quasi-static interaction would be expected.

The main result from the experiments with the C-terminal and N-terminal halves of OTF is that again two specific binding configurations are observed. They can be attributed to the low-pH and high-pH configurations of the intact protein. Thus the measurements clearly rule out the possibility that these binding configurations correspond to the C- and N-terminal metal binding sites of the protein, respectively. At both binding sites the ligand field changes when the pH value of the buffer solution is altered. The small deviations of the NQI parameters for the C-terminal half from the values for the intact protein or the N-terminal half, respectively, might be caused by the cleavage process used to generate the halves. No such effect has shown up in the N-terminal half, where the same NQI interaction parameters are observed as for the intact protein.

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