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Influence of protein dynamics on the metal-sites of ovotransferrin

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Abstract. Using the perturbed angular correlations (PAC) technique, the formation of hafnium-ovotransferrin complexes has been studied. Two binding configurations at each of the two specific binding-sites of the protein have been observed. They are characterized by well-defined electric quadrupole frequencies. Information about the dynamics of the protein was derived from temperature dependent measurements of the relaxation constant. The well-resolved spectra taken with fast BaF_2-detectors allow a precise determination of the relaxation behaviour of the protein. The results are compared with the predictions from a hydrodynamic model for the reorientation of macromolecules. Thus the hydrodynamic volume of ovotransferrin and its N-terminal half-molecule were determined. The ovotransferrin volume is in agreement with a value derived for human serum transferrin from small angle neutron scattering. From experiments with immobilized protein material there is evidence for internal protein dynamics which is probed by the Hf-ion bound to the specific metal-sites.

Key words: Ovotransferrin – Protein dynamics – Time differential perturbed angular correlations – Electric quadrupole interaction – Relaxation

Introduction

The transferrins are an evolutionary related group of glycoproteins with the property of reversibly binding iron and a series of other metal ions together with a bicarbonate anion. The latter is called the synergistic anion. Besides 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 aminoacids with a molecular mass of approximately 80 kDa. Crystallographic X-ray structure determinations of lactoferrin (Anderson et al. 1989, 1990) and TF (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). However, no crystallographic data are yet available for OTF. Each of the two lobes consists of two domains with one metal binding site in the interdomain cleft. The specific binding-site has high affinity for metabolic iron and other metals. 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 this metal which is essential for bacterial growth. For recent reviews on the transferrins see e.g. Brock (1985), Aisen and Harris (1989) and de Jong et al. (1990). Besides iron, many other metals bind to the transferrins. Representative for the binding of non-physiological metals, hafnium (Hf) was used for the studies reported here. It has been demonstrated previously that this metal binds specifically to the transferrins (Taylor et al. 1983).

For the study of the metal-binding to the specific sites of OTF the PAC method has been used. This is a nuclear spectroscopic technique similar to NQR or Mößbauer Spectroscopy. The specific metal-binding and the symmetry of the ligand field arrangement can be inferred from the electric field gradients (EFG) observed at the position of the probe nuclei. The high sensitivity of the method, along with the extremely good time resolution of the BaF_2-detectors, allows one to see subtle details of the ligand fields and their dependence on external conditions

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Abbreviations: PAC, perturbed angular correlations technique; TF, serum transferrin; LF, lactoferrin; OTF, ovotransferrin; OTF/2N, N-terminal half-molecule of ovotransferrin; NQR, nuclear quadrupole resonance; EFG, electric field gradient; NQI, nuclear quadrupole interaction; NTA, nitrilotriacetate; MES, 2-(*N*-morpholino)ethanesulphonic acid; HEPPS, *N*-2-hydroxyethyl-piperazine-*N*'-3-propanesulphonic acid; TRIS, tris(hydroxymethyl)-aminomethane

such as pH value and temperature. The essential advantage of the PAC method is, that in contrast to many other methods, the experiments can be performed under nearly physiological conditions: liquid samples were studied at room temperature with low concentrations of metal and protein in the order of nmol/ml. The isotope ^{181}Hf was chosen because it is a well-suited probe metal for the PAC method. As compared with measurements using ^{111}In (see Smith et al. 1984, Marsden et al. 1989), the hafnium-spectra are readily interpreted and more detailed information can be obtained, as will be shown in this paper.

Only limited information is available on the dynamics of the transferrins in solution. The dynamics of biological macromolecules comprises:

- large scale or collective motions like rotations of the whole molecule in a liquid, and
- internal motions and oscillations of parts of the molecule.

Thus the molecules do not exist in one conformation only as inferred from crystallography, but they appear in many conformational sub-states (Frauenfelder et al. 1979). In a series of studies it was shown that dynamic processes are essential for the function and specifity of the proteins and enzymes (see e.g. Karplus 1987).

The PAC method offers direct access to relaxation processes. Using the PAC isotope ^{181}Hf in combination with fast BaF_2-detectors a time resolution of 600 ps was achieved. Therefore it is possible to observe slow and fast relaxation processes in the time range from 10^{-6} s to 10^{-9} s. The PAC technique proved to be a valuable method for the study of protein dynamics in a wide temperature range for proteins in liquid solutions as well as for crystalline or frozen protein material.

The perturbed angular correlations technique

The PAC technique is based on the observation of an anisotropic correlation between the emission directions of two successive γ-rays while the probe nucleus is exposed to an electromagnetic perturbation. The electromagnetic interaction leads to a characteristic modulation of the correlation function for the γ-γ-cascade. A detailed description of the method is given by Frauenfelder and Steffen (1965).

The isotope ^{181}Hf decays with a comparatively long half-life of 42 days by β^--emission into ^{181}Ta. The half-life of the populated $I=1/2$ level ($T_{1/2}=18.1$ µs) is long enough for a complete rearrangement of the electron shell. Therefore, in contrast to the probe nucleus ^{111}In, no after-effects are observed with ^{181}Hf. It has been shown that these after-effects may lead to strong attenuations of the PAC spectra (Barfuß et al. 1982). The state with nuclear spin $I=1/2$ is populated with a branching of 93% and de-excites via a $\gamma(133\text{ keV}) \rightarrow \gamma(482\text{ keV})$ cascade to the ground state of ^{181}Ta. After the emission of the first γ-ray the excited $I=5/2$ level is left in a state where the levels with different magnetic quantum numbers m are unequally populated. This so-called alignment results in an anisotropic emission of the second γ-ray. For samples without microscopic ordering, e.g. polycrystalline or dilute material, the corresponding angular correlation function is given by

$$W(\Theta, t) = \sum_{\nu} A_{\nu\nu}\, G_{\nu\nu}(t)\, P_{\nu}(\cos\Theta), \quad \text{with even } \nu, \tag{1}$$

where the $A_{\nu\nu}$ are tabulated anisotropy coefficients that depend on the spins involved and the multipolarities of the emitted radiation. $P_{\nu}(\cos\Theta)$ are Legendre polynomials of the order ν with Θ being the angle between the emission directions of the two γ-rays. In the case of Hf only the nuclear quadrupole interaction (NQI) has to be taken into account. This interaction 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 $G_{\nu\nu}(t)$ of the correlation function. The quadrupole moment of the $I=5/2$ level is rather large and was taken as $Q=(2.36\pm0.05)$ barn from Butz and Lerf (1983). For the probe nucleus ^{181}Hf the approximation of only considering second order terms ($\nu=2$) is quite sufficient in most cases. When the electric fields are time independent, the static perturbation function explicitly reads

$$G_{22}^{\text{stat}}(t) = \sum_{k=0}^{3} s_{2k}(\eta)\cos(\omega_k t)\, e^{-\omega_k \delta t}, \tag{2}$$

where the $\omega_k = n_k(\eta)\,\omega_0$ are the observed angular frequencies. They reflect the characteristic EFG at the sites of the Hf-ions due to the electronic configuration of the molecular orbits. 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 Gaussian distribution. The amplitudes s_{2k} and the frequency ratios $n_k(\eta)$ were evaluated numerically from the matrix elements of the interaction Hamiltonian (see e.g. Bauer 1985). 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 $\mathbf{V}$ via the relation

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

where e is the unit charge, Q the nuclear quadrupole moment, and h Planck's constant. The asymmetry coefficient η reveals deviations of the ligand field arrangement from axial symmetry and is defined by

$$\eta = \frac{V_{xx} - V_{yy}}{V_{zz}} \quad \text{with} \quad 0 \le \eta \le 1. \tag{4}$$

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 corresponding amplitudes f_i give the fraction of nuclei that experience a specific EFG.

Owing 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. Therefore the theoretical perturbation function G_{22} has to be modified.

For tumbling macromolecules, the correlation time τ_c can be defined as the time during which the molecule changes its orientation by an angle in the order of 1 rad (Marshall 1978). If this characteristic correlation time τ_c is large compared with $1/\omega_0$ (the approximation of the so-called slow relaxation is valid for $\omega_0 \tau_c > 10$) then a quasi-static perturbation function G_{22} has to be used: The static perturbation function is superimposed by an exponential damping (Boyer and Baudry 1984) and in this time regime the relaxation constant λ is proportional to the inverse correlation time

$$G_{22}(t) = e^{-\lambda t} G_{22}^{\text{stat}}(t) \quad \text{with} \quad \lambda = \frac{1}{\tau_c}. \tag{5}$$

However, in the case of rapid fluctuations, i.e. when the correlation time τ_c is small compared with $1/\omega_0$ (the approximation of the so-called fast relaxation is valid for $\omega_0 \tau_c < 0.1$), the time dependence of the perturbation function reduces to an exponential decay and the relaxation constant is directly proportional to the correlation time (Boyer and Baudry 1984)

$$G_{22}(t) = e^{-\lambda t} \quad \text{with} \quad \lambda = 2.8\left(\frac{\eta^2}{3} + 1\right)\omega_0^2 \tau_c. \tag{6}$$

In liquid samples with macromolecules of molecular mass of about 80 kDa such as OTF the rotational movement is rather slow and the correlation time consequently long. Therefore a slow relaxation behaviour was expected for the measurements with liquid OTF samples. It has to be noted that in the case of slow relaxation the dynamics are completely decoupled from the quadrupole interaction in the sense that no dependence of λ on ω_0 exists.

Experimental

Sample preparation

The apo-form of hen OTF was purchased from SERVA (Heidelberg, FRG). The C- and N-terminal half-molecules were derived and isolated according to the procedure described by Oe et al. (1988). For pH values up to 8.0 the freeze dried protein 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. As in the case of TF (Taylor et al. 1988) the presence of phosphate is necessary for observing the characteristic binding of Hf to the metal-sites. At higher pH values a 100 mM TRIS buffer was used. The OTF protein concentration was 25 nmol/ml and the total sample volume amounted to 0.5 ml.

Hafnium chloride solution with a specific activity ranging from 700 to 1500 MBq/mg ^{181}Hf in 2 M HCl was purchased from Amersham-Buchler (Braunschweig, F.R.G.). The Hf-ions were complexed with a fourfold 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 about 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 (Taylor et al. 1988) was used to establish that the Hf-ions were specifically bound to OTF. For some experiments the protein was immobilized in order to disable the reorientation of the free molecules. In this case the protein was bound covalently to a matrix using cyanogen-bromide-activated (CNBr) Sepharose 4B, according to the procedure described by van Eijk and van Noort (1976). The fraction of protein molecules that did not bind to the matrix was determined by measuring the free ^{181}Hf activity in the samples and was found to be less than 5%. The free, mobile OTF molecules and the non-protein bound Hf-ions were removed by decanting the surplus of buffer above the gel.

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 the loss of CO_2 the samples were sealed hermetically. An incubation time of at least 12 h was allowed before the PAC measurements were started. The experiments were performed at different temperatures between 0 °C and 45 °C. The temperature of the samples was stabilized and checked using a Lauda compact thermostat (Lauda Königshofen, FRG) and the deviations from the required temperature were found to be less than 0.2 °C. The recording time for a single spectrum was between 24 h 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. No changes of the spectra were observed, thus proving the stability of the samples.

PAC spectrometer

A conventional four-detector system was used to measure the PAC spectra. Owing to the comparatively high frequencies observed in the transferrins (in the order of GHz), the spectrometer had to be optimized for extreme time resolution. Four cylindrical $\varnothing$ 40 mm × 40 mm BaF_2 single crystals together with specifically designed photomultiplier-bases for the Valvo XP2020Q tubes were used. A time resolution as good as 600 ps (FWHM) was achieved for the 133 keV–482 keV cascade in ^{181}Hf. The coincidence spectra $N(\Theta, t)$ were taken as a function of the delay time t between the emission of the two γ-rays under fixed angles $\Theta = 90°$ and $\Theta = 180°$. Details of the setup are described elsewhere (Heidinger et al. 1987). The excellent time resolution is necessary to distinguish between correlated parameters such as the frequency distribution width δ and the relaxation constant λ. Both quantities parameterize an exponential damping of the spectra in the timescale. But only the damping due to δ depends implicitly on the frequency.

Data analysis

Eight coincidence spectra $N(\Theta, t)$ were recorded simultaneously with an 8 k multichannel analyzer and the data were transmitted to a MicroVAX II minicomputer. The coincidence spectra $N(\Theta, t)$ are proportional to the correlation function $W(\Theta, t)$ and therefore the PAC signal $R(t)$ is obtained by forming the ratio

$$R(t) = 2\,\frac{N(180°, t) - N(90°, t)}{N(180°, t) + 2N(90°, t)} = A_{22}^{\text{eff}}\, G_{22}(t)\,. \qquad (7)$$

The effective anisotropy coefficient A_{22}^{eff} of the emitted γ-γ-cascade depends on the spins of the nuclear levels involved, the multipolarities of the γ-rays and the geometry of the experimental setup. The theoretical value of $A_{22}^{\text{eff}} = -0.23$ was verified experimentally.

The parameters of the NQI were determined from the $R(t)$-spectra using a least-squares fit algorithm. The significance of a parameter value extracted by this method is reflected in the corresponding parameter error. However, correlations between parameters and numerical implications during the least-squares iterations may shift the fit values and their errors. It was therefore checked how precisely the relaxation constant, the primarily discussed parameter in this paper, can be determined. The results are given in Fig. 1. The reduced sum of the squares of deviations χ^2 was calculated for the optimum fit and plotted as a function of λ. Each data point corresponds to a least-squares fit where λ was fixed to a certain value, whereas all other parameters remained unconstrained. The calculations were performed for two typical and representative ^{181}Hf-OTF spectra taken at pH 7.5 and pH 9.0. The horizontal line in Fig. 1 gives the 1.01 χ^2_{min} value, which was taken as the significance level. The graphs show a well-defined minimum and a comparatively narrow distribution of χ^2 as a function of λ for both configurations. Therefore the fit gives a reliable value for the relaxation constant. The high-pH data are slightly more precise and the value of the relaxation constant of f_2 is about 10 MHz higher than for f_1. Furthermore an influence of λ on δ has shown up for both configurations: The optimum fit value of δ changes linearly with the relaxation constant λ, as can be deduced from Fig. 1. The slope is found to be larger for the low pH configuration f_1. Therefore the uncertainty in the determination of λ will be smaller for the second configuration f_2.

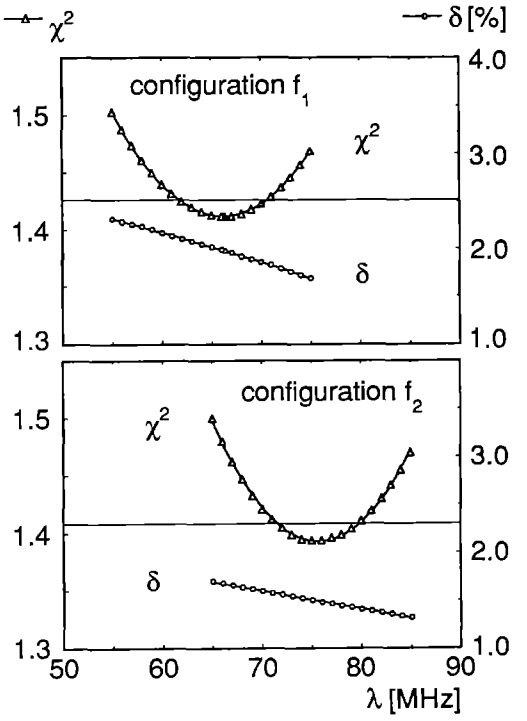

Fig. 1. Reduced sum of squares of deviations χ^2 (left y-axis) and frequency distribution width δ (right y-axis) plotted as a function of the relaxation constant λ for typical ^{181}Hf-OTF spectra taken at pH 7.5 and pH 9.0 and at a temperature $T = 35$ °C. For the high-pH configuration f_2 the relative width of the χ^2 curve is slightly smaller and the relaxation constant λ at χ^2_{min} is significantly higher than for the low-pH configuration f_1. The dependence of δ on λ is more pronounced for f_1. Increasing the relaxation constant λ causes a decrease of the frequency distribution δ in both cases

Results

In a series of systematic experiments hafnium labelled OTF samples have been studied. The specific binding of the ^{181}Hf-ions to the metal-sites of OTF is reflected in the PAC spectra. As in the case of TF (Taylor et al. 1988), the main parameters that influence the EFGs are the pH value and the temperature of the samples. Representative PAC spectra for samples prepared at pH 7.5 and pH 9.5 are given in Fig. 2. Dependent on the conditions of the preparation three different EFGs were measured. Two of them are characterized by very small values of the frequency distribution width δ of about 2%. According to their appearance they are termed the low-pH (f_1) and the high-pH (f_2) configuration. These two EFGs have to be attributed to well-defined binding configurations of the Hf-ions. The frequencies and the asymmetry parameters of the fractions f_1 and f_2 are quite different, thus proving that the ligand field arrangement changes when the pH value is altered.

For the fit of the theoretical model function to the data a third fraction f_3 with a small amplitude has to be taken into account. Its frequency distribution width turns out to be rather broad ($\delta \approx 16\%$), indicating a binding of these Hf-ions in several slightly different configurations. The percentage of probe nuclei responsible for this third fraction was between 10% and 20%. The relaxation constant for the third configuration could not be determined independently and it was assumed to be identical to the relaxation constant of the dominant configuration f_1 or f_2, respectively.

In Table 1 the NQI parameters are listed for the three fractions. The small values of the frequency distribution width δ for fractions f_1 and f_2 allow a precise determination of the relaxation parameter λ. The spectra were best described by an axially symmetric EFG for fraction f_3 (i.e. $\eta = 0$).

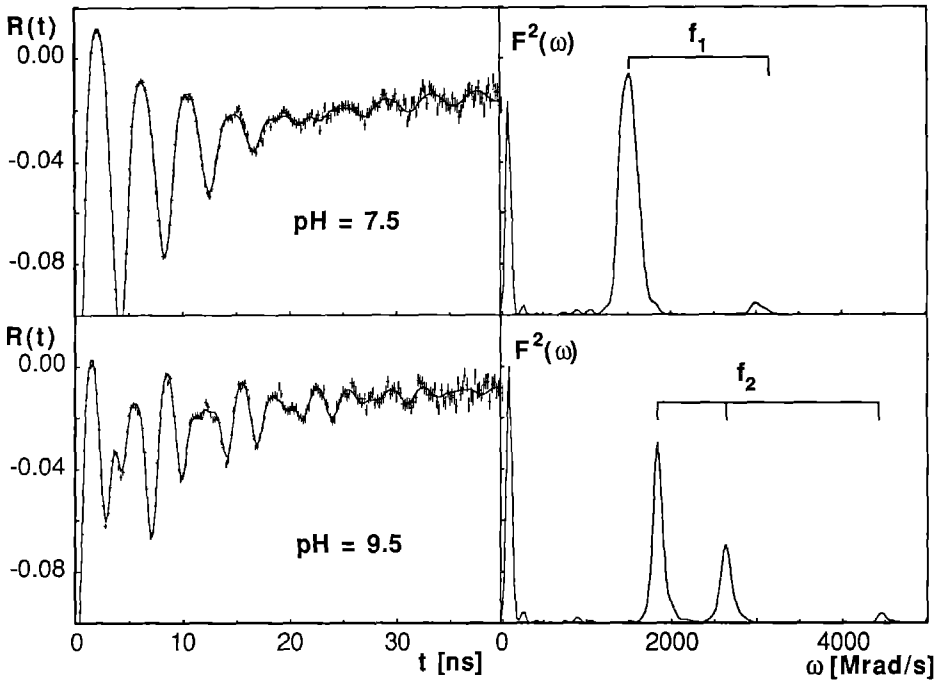

Fig. 2. The $R(t)$-spectra and the squared Fourier-amplitudes $F^2(\omega)$ of ^{181}Hf-OTF taken with samples at pH 7.5 and pH 9.5 show characteristic signals for the configurations f_1 and f_2, respectively

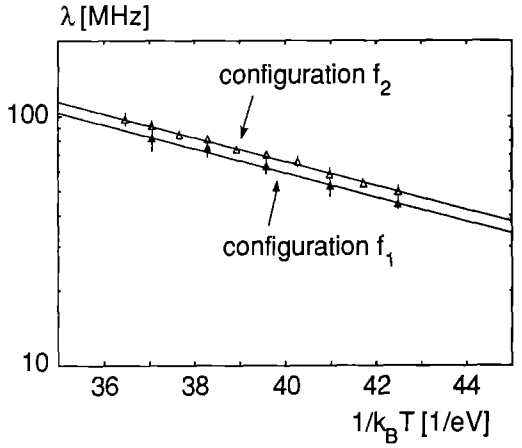

Fig. 3. Arrhenius plots of the relaxation constant λ for ^{181}Hf-OTF at pH 7.5 (configuration f_1) and pH 9.5 (configuration f_2). Both configurations are characterized by slow relaxation. The corresponding activation energies are $E_a = (0.114 \pm 0.004)$ eV for f_1 and $E_a = (0.112 \pm 0.003)$ eV for f_2.

Table 1. NQI parameters[a] for ^{181}Hf-OTF measured at 25 °C

	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

The PAC spectra for the OTF samples were recorded in a temperature range between 0 °C and 45 °C. The frequency distribution width and the asymmetry parameter show only slight variations with temperature for both configurations. The changes of these parameters are in the order of the parameter errors and are not regarded as significant. In contrast, the relaxation constant displays a marked variation with temperature (see Fig. 3). The dynamics of the protein are therefore strongly influenced by temperature. For relaxation processes with an activation energy E_a, the transition rate W or inverse correlation time is given by an Arrhenius relation (Shinar et al. 1984)

$$W = \frac{1}{\tau_c} = W_0 \, e^{-E_a/k_B T} \tag{8}$$

with k_B being the Boltzmann constant.

Within the observed temperature range the logarithm of the relaxation constant λ is proportional to $1/k_B T$ with a negative slope. This behaviour indicates that the assumption of slow relaxation for the binding of Hf to OTF is justified. This is verified by the product $\omega_0 \tau_c$ which has a value between 10 and 20 for both fractions. Over the whole temperature range the relaxation constant for fraction f_2 is approximately 5 to 10 MHz higher than for fraction f_1. The slope, however, remains unchanged.

It is interesting to compare the relaxation data for OTF with data for OTF half-molecules, with still intact metal-sites. The NQI parameters of hafnium in the N-terminal half-molecule (OTF/2N) have been investigated as a function of temperature, as reported earlier (Appel et al. 1990). Both characteristic binding states were observed with the same NQI parameters as for the intact protein. The relaxation was studied for temperatures between 0 °C and 40 °C using N-terminal half-molecules at pH 9.2. For this high-pH configuration the relaxation constant turned out to be clearly larger than for the intact protein. This finding is in accordance with the expectations: The smaller molecular volume of the OTF fragment should result in a smaller correlation time τ_c and thus in a larger relaxation constant. The value at a temperature of 25 °C is $\lambda = (98.1 \pm 6.1)$ MHz for the half-molecule and $\lambda =$

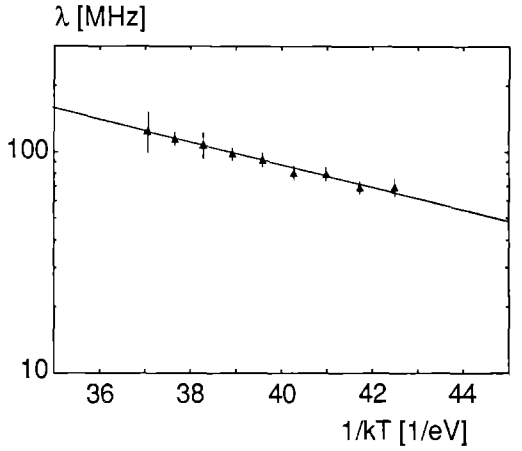

Fig. 4. Arrhenius plot of the relaxation constant λ for the N-terminal OTF fragment at pH 9.2. The binding configuration is characterized by slow relaxation. The corresponding activation energy is $E_a = (0.116 \pm 0.007)$ eV

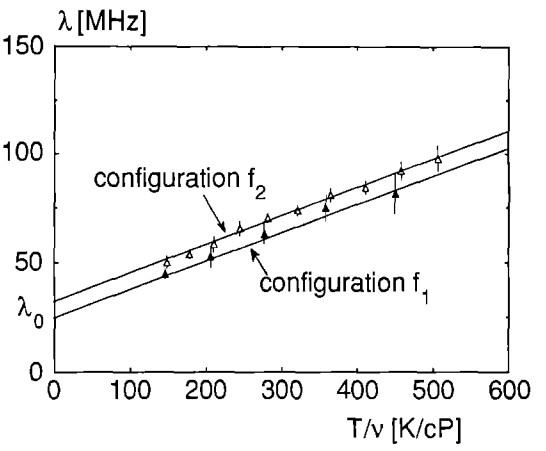

Fig. 5. Debye plot of the relaxation constant λ as a function of T/v (v: measured shear viscosity of the samples) for ^{181}Hf-OTF at pH 7.5 (configuration f_1) and pH 9.5 (configuration f_2). The extrapolation of the data to $T/v = 0$ leads to a non-vanishing relaxation constant $\lambda_0 \approx 30$ MHz

(68.2 ± 1.9) MHz for the intact OTF molecule. Since the weight and the volume of the half-molecules are still rather large, slow relaxation was expected also for OTF/2N. A graphic representation of the relaxation constant for the half-molecules as a function of temperature is given in Fig. 4.

The agreement between the theoretical dependence and the experimental data in the Arrhenius representation is excellent and the negative slope justifies the model of slow relaxation also for the N-terminal half-molecule. The absolute values of the relaxation constants are about a factor 1.4 higher than for the intact molecule. The activation energies for the dynamic processes in the intact protein, deduced from the slopes of the Arrhenius plots in Fig. 3, were found to be identical within the parameter errors: $E_a = (0.114 \pm 0.004)$ eV for f_1 and $E_a = (0.112 \pm 0.003)$ eV for f_2. The same activation energy $E_a = (0.116 \pm 0.007)$ eV was determined for the N-terminal half-molecule despite the fact that the absolute value of the relaxation constant is clearly different. These values of the activation energy compare favourably with the value of 0.10 eV for rat TF (Appel et al. 1987). They are significantly higher than the value of $E_a = (0.067 \pm 0.004)$ eV for human TF, observed in ^{111}In-PAC experiments (Smith et al. 1984, Marsden et al. 1989). As expected the relaxation constants observed in the transferrins are in the same order for the probe metals indium and hafnium.

Discussion

As shown for TF (Taylor et al. 1988) two specific and non-identical binding configurations with different NQI parameters have also been observed for the binding of ^{181}Hf to the specific sites of OTF. For half-molecular OTF fragments it has been concluded that the two binding configurations cannot simply be attributed to the two binding-sites in the protein, because both binding-sites are capable of forming the two configurations f_1 and f_2 (Appel et al. 1990). A change of the pH value induces alterations of the ligand field arrangement at both metal-sites of the protein. The small frequency distribution width observed for the configurations f_1 and f_2 allows one to determine the relaxation parameters accurately.

Assuming that the relaxation is caused by the reorientation of the whole molecule, the hydrodynamic volume can be calculated: The Debye model (Debye 1945) for rotational diffusion of spherical or approximately spherical molecules in a fluid of viscosity v sets up a relation between the correlation time τ_c and the volume

$$\tau_c = Vv/k_B T. \tag{9}$$

In this hydrodynamic model stick-boundary conditions are assumed which couple molecular rotations to the solvent at the surface of the molecules. It has long been known that this model is especially successful in predicting correlation times of macromolecules (Tanford 1961). It was therefore used to determine the hydrodynamic volume of OTF from the experimental relaxation values.

Figure 5 shows the relaxation constant as a function of T/v. The dynamic viscosity v of the samples was measured as a function of temperature using an Ubbelhode micro-viscometer. The linear function $\lambda = \lambda_0 + AT/v$ was fitted to the data with A being the proportionality constant and λ_0 taking care of the offset at $T/v = 0$. A rather large intercept λ_0 of about 30 MHz was deduced from the data. In a straightforward calculation of the volumina using the Debye relation a pronounced temperature dependence was found. The results range from 40 to $80 \cdot 10^{-27}$ m^3. They are, as compared with the values taken from neutron scattering experiments (Martel et al. 1980), up to a factor of three too low. Both facts, the large value for the offset and the markedly too low value for the molecular volume have lead to the assumption that fluctuating field gradients occur in the Hf-OTF system. They are not caused by the reorientation of the whole protein, but have to be attributed to motions of parts of the molecule or to internal protein dynamics.

In order to test this hypothesis, experiments with immobilized protein molecules were performed. The covalent binding of the OTF molecules to a high molecular polymer is a method of choice for avoiding the reorientation of the protein molecules in the liquid phase. To the authors this appears to be the least invasive method as compared with freezing, freeze-drying or crystallizing. The NQI parameters measured with the immobilized protein samples are given in Table 2. Both specific configurations f_1 and f_2 are observed with no significant changes of the NQI parameters as compared with the mobile protein. It can be deduced that the immobilization has no influence on the specific binding-sites and their symmetry. The only parameter that is influenced by this procedure is the relaxation constant which is clearly reduced. Obviously it does not vanish, as one would expect, if the only relaxation mechanism were the reorientation of the protein molecule. We conclude that the relaxation at room temperature observed for the two configurations of immobilized OTF has to be attributed to internal protein dynamics.

The relaxation constant of the immobilized protein was measured as a function of temperature. It is compared with the relaxation constant of the mobile protein and its N-terminal fragment in Fig. 6. The offset $\lambda_0 \approx$ 30–40 MHz is observed in all three samples and it becomes evident that this intercept λ_0 at $T/v=0$ corresponds to the internal dynamics of the protein. Even in the case of the immobilized protein the relaxation constants show a slow relaxation behaviour and the λ value for the binding configuration f_1 is markedly smaller than for f_2. Based on the comparatively long correlation times it may be speculated that these internal dynamics are due to oscillations of the two domains in each lobe. They affect the EFG at the metal site located in the cleft between these two domains.

The difference between the relaxation constants of the mobile and immobilized protein has to be attributed to the reorientation of the OTF molecules. For both configurations this difference has a value of about 30 MHz at 25 °C (35 ± 2 MHz for f_1, and 30 ± 3 MHz for f_2) which corresponds to a typical rotational correlation time of $\tau_c \approx 33$ ns. The hydrodynamic volume of the protein molecule was recalculated for all temperatures measured here using the reorientational part of the relaxation only. The corrected molecular volume was calculated from the slope of the Debye plot: $V=(149 \pm 17) \cdot 10^{-27}$ m^3 for configuration f_1 and $V=(129 \pm 6) \cdot 10^{-27}$ m^3 for configuration f_2. This finding is in good agreement with a molecular volume of $V=(144 \pm 45) \cdot 10^{-27}$ m^3 determined by small angle neutron scattering experiments (Martel et al. 1980) for human TF. The volume of the N-terminal half-molecule was calculated in a similar way assuming that the internal dynamics are identical for the intact protein and the half-molecule. The calculations yielded a volume for OTF/2N of $V=(84 \pm 6) \cdot 10^{-27}$ m^3.

However, it has to be noted that the values V calculated here are connected to the effective volume V^{eff} of the hydrated molecule via a proportionality constant $\varkappa = V/V^{\text{eff}}$ with $0 \leq \varkappa \leq 1$. This correction $\varkappa$ takes into account the specific solute-solvent interactions (Baudry et al. 1976, 1981). These are, for example, deviations from the stick-boundary conditions and deviations of the molecular shape from spherical symmetry. Perrin (1936) has calculated the correction for prolate and oblate rotational ellipsoids. The axis ratios for iron loaded TF (Gorinsky et al. 1979) and OTF (Abola et al. 1982) molecules derived from other methods are of the order 1:1.5. For axis ratios of 1:2 the correction has a value of $\varkappa \approx 0.7$ for prolate and $\varkappa \approx 0.9$ for oblate shapes of the molecules. The effective volume of the OTF molecule is therefore expected to be somewhat larger than the measured hydrodynamic volume.

The ratio of the measured volumes of the intact protein and its N-terminal half calculated for fraction f_2 is 1.55 and deviates from the expected value of 2. It may be speculated that the shapes and axis ratio for OTF and OTF/2N are non-identical thus causing the deviation mentioned above.

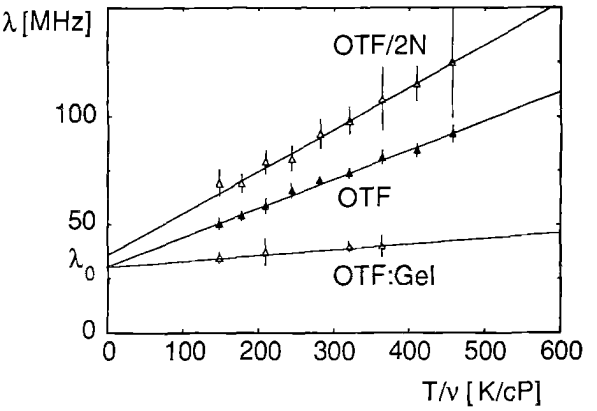

Fig. 6. Debye plots of the relaxation constant λ as a function of T/v (v=shear viscosity) for ^{181}Hf-OTF, the N-terminal half-molecule ^{181}Hf-OTF/2N and the immobilized protein ^{181}Hf-OTF:Gel at pH 9.2. The offset λ_0 (OTF/2: (38.2 ± 2.3) MHz; OTF: (31.1 ± 1.4) MHz; OTF:Gel: (31.5 ± 1.0) MHz) is caused by the internal dynamics of the protein

Table 2. NQI parameters[a] for immobilized ^{181}Hf-OTF measured at 25 °C

	ν_Q [MHz]	η	δ [%]	λ [MHz]
f_1	928.8 ± 2.1	0.877 ± 0.002	3.5 ± 0.2	24.5 ± 1.1
f_2	1478.3 ± 1.3	0.581 ± 0.001	1.2 ± 0.2	38.5 ± 2.1
f_3	938.4 ± 22.8	0	17.7 ± 2.6	as for f_1 or f_2

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

Conclusions

The PAC technique has been shown to be a valuable method for the investigation of both the metal-sites and their dynamics in biological macromolecules. The experiments prove that the relaxation in OTF is composed of reorientation of the whole molecule and of internal dynamics. The pH value of the solution has some influence on the internal dynamics which seem to be related to the

local conformation of the metal-site. No drastic effect of pH on the reorientation of the molecules was detectable. The volumina deduced from the experiments are within the parameter errors identical for low and high pH values. Thus no significant variation of the volume due to conformational changes can be deduced from the data. All relaxation effects, the reorientation of the protein molecule in solution but also the internal dynamics are well-described by the model of slow relaxation.

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