Metal Site Structure in a Protein Studied by Differential Perturbed Angular Correlations

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A static quadrupole interaction has been determined for the 247-keV state of ^{111}Cd bound to the active site of human carbonic anhydrase B. The rotational diffusion was slowed down by dissolving the enzyme in a 46% sucrose solution at 22°C. The result yields two different frequencies and asymmetries, indicating either two forms of the enzyme or an additional site for Cd.

The structure of many biological macromolecules containing metal ions (metalloproteins) has been studied by means of a variety of physical methods including electron-spin resonance, nuclear magnetic resonance, Mössbauer spectroscopy, and x-ray diffraction.[1] For this group of proteins the metal ions play an essential role in their function[2]; hence it is important to study the structure-function relationship for the metal-containing groups of these proteins.

Differential perturbed angular correlations (DPAC) have previously been used in the study of rotational correlation times for proteins in solution.[3] In this paper the application of DPAC is introduced for the study of local protein structure. This method has the advantage of being applicable to solutions as well as crystals, and to be especially sensitive to the local charge asymmetry in the proteins. In the case of the enzyme carbonic anhydrase chosen for this investigation, many experimental data are available including x-ray diffraction.[4] Nevertheless, there are still many unanswered questions about the structure and function of this enzyme, especially concerning the enzyme in solution.[5]

The ^{111}Cd 49-min isomer used in this experiment decays through the spin sequence

$$\tfrac{11}{2}\xrightarrow{\gamma_1\,(E3)}\tfrac{5}{2}\xrightarrow{\gamma_2\,(E2)}\tfrac{1}{2},$$

where $\gamma_1 = 150$ keV and $\gamma_2 = 247$ keV.[6] The angular correlation resulting from randomly oriented interactions has for this decay the form[7]

$$W(\theta,t)=\exp(-t/\tau_N)[1+A_2G_2(t,I,\omega_0,\eta,\tau_R)(3\cos^2\theta-1)/2], \tag{1}$$

where θ is the angle between the two γ rays; $I = \frac{5}{2}$ is the spin and $\tau_N = 122$ nsec is the lifetime of the intermediate state; A_2 is a constant determined by the spin of the three states and the multipolarity of the two γ rays, and is equal to 0.179 in this case ($A_4 \sim 0$).[6] $G_2(t,I,\omega_0,\eta,\tau_R)$ is a factor which represents the interaction with the surroundings determined by the parameters

$$\omega_0=\frac{6eQV_{zz}}{\hbar 4I(2I-1)},\quad \eta=\frac{V_{xx}-V_{yy}}{V_{zz}},$$

and τ_R, where Q is the quadrupole moment of the intermediate state, V_{xx}, V_{yy}, and V_{zz} are components of the field gradient in the principal system, and τ_R is the relaxation time. The derivation of G_2 for a static interaction (Fig. 1) can be found in Ref. 7. In the case of ^{111}Cd bound to carbonic anhydrase dissolved in water,[3] it seems that relaxation effects quickly produce disalignment of the nuclear spin. This is expected if $\omega_0\tau_R \sim 1$, which is supported by the calculation of G_2 presented by Winkler and Gerdau.[8] Thus in order to see structure in the time spectrum it is necessary to have a situation where $\omega_0\tau_R \gg 1$. In the case of a macromolecule dissolved in a liquid

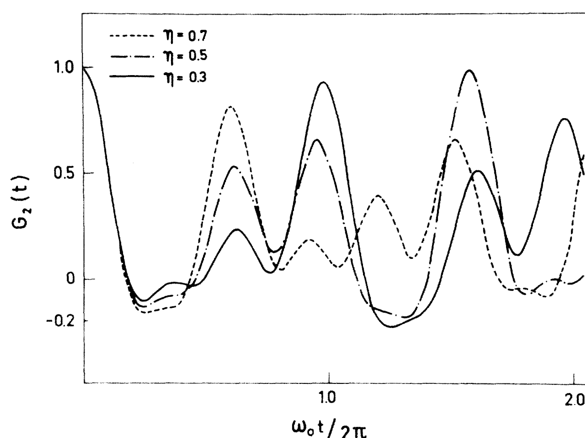

FIG. 1. The perturbation factor G_2 for a static quadrupole interaction and $I=\frac{5}{2}$ for a few values of η (others can be found in Ref. 7).

medium, it appears reasonable to assume that a relaxation effect is produced by a rotational diffusion of the macromolecules. These approximations yield for spherical molecules[9]

$$G_2 = \exp(-t/\tau_R)G_{2,\text{ static}}\,. \qquad (2)$$

A situation where $\omega_0\tau_R \gg 1$ can be reached if one uses a more viscous solvent than water. This is confirmed by the simple Debye theory of polar liquids, where the relaxation time is given by

$$\tau_R = 4\pi a^3\eta/3kT. \qquad (3)$$

Here η is the viscosity and a is the molecular radius.

The ^{111}Cd isomer at 397 keV was produced by the reaction ^{108}Pd$(\alpha,n)^{111}$Cd using α particles at 18 MeV. No other γ lines besides 247 and 150 keV were observed in the energy spectrum. Two independent measurements have been performed on equivalent samples. The weights of the two metallic Pd targets amounted to 2 and 30 mg, respectively. The targets were dissolved in freshly prepared hot aqua regia, evaporated to dryness and redissolved in 0.1N HCl. The main part of the Pd ions was removed by extraction with a 0.05% dithiozone solution in carbon tetrachloride. The ^{111}Cd activity was then isolated by a similar dithiozone procedure,[10] giving the divalent Cd ions in 0.1N HCl. The pH of the final 1.5-ml sample was adjusted to 6.5 by 1N NaOH and 50 μl of 0.55M 2-(N-morpholino) ethane sulphonic acid (MES).

Human carbonic anhydrase B (HCAB) is a metalloenzyme which contains 1 gram atom Zn per molecule. The apoenzyme was prepared as described by Coleman.[11] The residual Zn content of the apoenzyme preparation was less than 6%. The apoenzyme was dissolved in 0.055M MES pH 6.5 buffer. The concentration of the enzyme was determined, by measuring the absorption at 280 nm, to be $2.9\times10^{-4}M$, using a molar extinction coefficient of 4.65 mole^{-1} cm^{-1} based on a molecular weight of 28.800.[4] To the 1.5-ml buffered Cd^{++} solution was added 100 μl of the apoenzyme solution. 1.5 ml of the Cd-HCAB solution was then added to a 60% solution of sucrose in 0.007M MES pH 6.5, giving a final sucrose percentage of 46.

Precautions to prevent contamination by adventitious metal ions were taken throughout,[12] and

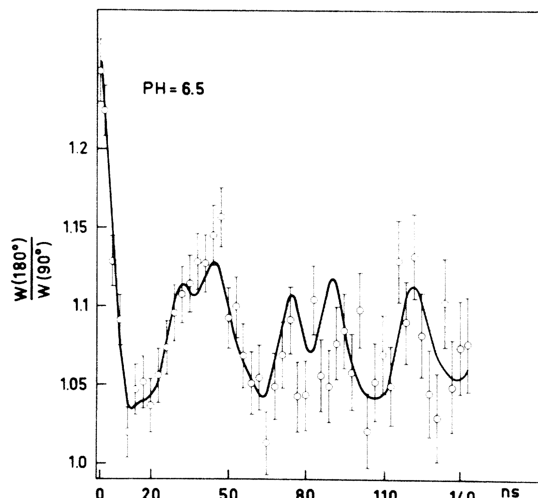

FIG. 2. Added time spectra for ^{111}Cd-HCAB dissolved in a 46% sucrose solution at 22°C. The full drawn curve represents a χ^2 fit to the experimental points.

the ratio of Pd atoms to enzyme molecules was verified by a separate tracer experiment to be less than 1:100.

Three NaI detectors at fixed angles 0, 90, and 180° in connection with a slow-fast coincidence setup were used in recording the time spectra. The same time-to-amplitude converter was used for both 0-90° and 0-180° coincidence spectra and the signals corresponding to different spectra were routed to appropriate memory sections of the multichannel analyzer by energy coincidence signals.

The time spectrum is shown in Fig. 2 together with a least-squares fit to the data. The ordinate scale gives $W(180°,t)/W(90°,t)$ without normalization. The time resolution is better than 4 nsec, and we have therefore not corrected the least-squares fit for finite time resolution. The relaxation time τ_R can only crudely be estimated from the data ($7\times10^{-8}\leq\tau_R\leq6\times10^{-7}$ sec) and we have instead used the relaxation time calculated by the Debye formula (3) which gives $\tau_R = 125$ nsec, using 23.5 Å as an effective molecular radius,[13] $T = 295$°K, and $\eta = 9.3$ cP. A reasonable fit is only possible with the sum of two different quadrupole interactions. The function used in the least-squares fit is approximated [see Ref. 3, Eq. (2)] by

$$W(180°)/W(90°) = a + bG_2(\omega_0^{\,2}, \eta_2, \tau_R) + c[G_2(\omega_0^{\,1}, \eta_1, \tau_R) - G_2(\omega_0^{\,2}, \eta_2, \tau_R)],$$

where c/b and $1-c/b$ are equal to the percentage of ω_0^1 and ω_0^2, respectively. For the perturbation factor G_2 we used Eq. (2). The χ^2 surface was searched for the absolute minimum in the parameters ω_0^1, η_1, ω_0^2, and η_2, each time making a linear fit to the parameters a, b, and c. The result gives

$$\omega_0^1 = 108 \pm 12 \text{ MHz},$$

$$\omega_0^2 = 135 \pm 10 \text{ MHz},$$

$$\eta_1 = 0.6 \pm 0.2,$$

$$\eta_2 = 0.4 \pm 0.2,$$

$$c/b = (45 \pm 35)\%,$$

$$1 - c/b = (55 \pm 35)\%.$$

The aim has been to show that it is possible to determine a quadrupole interaction in a protein by using DPAC. The results are encouraging and seem to confirm the Debye model for relaxation, giving that the relaxation time is considerably enhanced in a 46% sucrose solution relative to the solution used in Ref. 3. Evidence for binding to the active site for Cd can be taken from Ref. 11 where Coleman has found no Zn binding to the Cd enzyme within several weeks. Concerning the effect of sucrose on the enzyme, no evidence of conformational changes has been found for HCAB in a 20% sucrose solution.[14] Furthermore, we have measured the visible absorption spectrum of the cobalt enzyme, with and without 46% sucrose, and in both cases the spectra are identical to those published by Whitney,[15] suggesting that there can only be small changes in the metal coordination by the addition of sucrose. A more detailed study of the effect of sucrose, using DPAC, is currently investigated.[16]

It is interesting to note that the best fit to the time spectrum is obtained with two different quadrupole interactions, indicating either two forms of the enzyme or an additional site for Cd. The latter possibility cannot be ruled out yet, but a measurement similar to the one presented here, performed on the native zinc-containing enzyme instead of the apoenzyme, should reveal whether there is a Cd site different from that of the active site. If the former possibility is the case, it would, even though the Cd enzyme has no catalytic activity, be of great interest to study the pH dependence of the quadrupole interaction, because one might then be able to correlate the pH profile of the catalytic activity to a possible equilibrium between two forms of the enzyme.[5] Further studies of this possibility are in progress.[16] It is also interesting to note that the quadrupole interaction here determined indicates a rather asymmetric metal coordination.

Other new possibilities with this method are a comparative study of proteins in crystal and liquid phases and a study of the local dynamic behavior of proteins in solution.

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