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^{13}C-NMR STUDY OF BOVINE CARBONIC ANHYDRASE *B* RELAXATION DYNAMICS IN SOLUTION AND IN THE SOLID STATE

(*Presented by E. Lippmaa*)

^{13}C-NMR relaxation is a powerful tool for the study of intramolecular dynamics in macromolecules. In the case of protonated carbons in small rapidly rotating molecules dipolar interaction leads to exponential free induction decay with a single relaxation time. In macromolecules, where many different motions over a wide time range are superimposed, other relaxation mechanisms and relaxation crossterms may lead to nonexponential relaxation. The relaxation of ^{13}C nuclei of macromolecules in solution has generally been taken to be exponential. This is by no means obvious and can only be true in the case of internal segmental motion involving all carbons. If the whole molecule or the main hydrogen-bonded domains behave motionally as solid blocks, strong nonexponentiality would be expected. The question of relaxation exponentiality has also been under study in the case of dielectric and mechanical relaxation. Various empirical fitting functions for data analysis were used, but causes of the nonexponentiality experimentally observed remain open.

This work was intended to clarify the spin-lattice relaxation behavior of enzyme macromolecules in solution. For comparison purposes solid state ^{13}C-NMR relaxation was studied, using powder samples where nonexponential relaxation could be expected. The so-called relaxation spectra for all resonances in the investigated spectra were calculated using a nonlinear least-squares algorithm. A quantitative measure for relaxation exponentiality is proposed and is shown to be different for the same enzyme in solution and in the solid state.

Experimental

Bovine carbonic anhydrase *B* (E.C.4.2.1.1) was prepared as previously described [1]. The spin-lattice relaxation time T_1 of carbon nuclei in solution was measured at 125.76 MHz on the «Bruker» AM-500 NMR spectrometer by the inversion recovery with Fourier transform (IRFT) technique. Eight delay times (t=20, 60, 150, 400, 800, 2000, 4000, and 6000 ms) with a relaxation delay of 6 s were used. Broadband proton decoupling by the WALTZ-16 scheme [2] and broadband inversion using composite 7-step π pulse proposed by R. Tycko [3] were used. The temperature was stabilized at 13 °C, using intense air-cooling (1000 l/h) to avoid thermal denaturation.

The solid state relaxation experiment with a powder sample (200 mg of dry lyophilized carbonic anhydrase *B*) was carried out on a «Bruker» CXP-200 NMR spectrometer at 50.31 MHz, using a home-built magic-angle spinning probehead with a 7 mm O.D. double-bearing rotor and a

spinning rate of 3.5 kHz. Relaxation in powder sample was measured at room temperature using 15 delay times, and polarization transfer during 1 ms from the ^1H nuclei was used.

All computations were done on an Aspect computer and the programs were written in Pascal.

Computation of relaxation times

A method to calculate the relaxation time constant and to check relaxation exponentiality in the case of spectra with many overlapping resonances was developed. Existing programs are intended to work in the case of well-resolved resonances. The computation method generally used is steepest descent, which is slowly converging and time-consuming. For faster processing, a method of linearization was used and the derivation of formulas for the special case of IRFT experiment is given in the Appendix. Relaxation times were calculated for every data point on the chemical shift axis to obtain the so-called relaxation spectrum. It is evident that relaxation spectra provide better insight into the dependence of relaxation times on chemical shifts over the spectral region investigated, and also give an estimate of experimental error even in the case of poor S/N ratio. In the case of an ideal IRFT experiment, integration of the differential equation describing the recovery of the initial nuclear magnetization M_0 leads to the variation of magnetization M with time t according to

$$M(t) = M_0[1 - 2\exp(-t/T_1)], \quad (1)$$

where T_1 is the time constant of magnetization recovery. The main reasons for experimental imperfections which cause deviations from this equation are: a deviation from the exact π-pulse setting, incomplete recovery between succeeding pulses, and spectral offset effects. It has been shown by M. Sass and D. Ziessow [4] that one of the ways to overcome nonidealities in the IRFT experimental data analysis is nonlinear least-squares 3-parameter fit to the formula

$$I(t) = A - B\exp(-t/T_1), \quad (2)$$

where $I(t)$ is the signal intensity at relaxation delay t, A and B are parameters. In addition to those parameters the coefficient of magnetization inversion $K(\Delta\omega) = B/2A$ as a function of spectral offset $\Delta\omega$ can also be calculated. For an ideal IRFT experiment, A is the signal intensity I_0, corresponding to a value of magnetization at time $t=0$, B is the equilibrium intensity I_∞ at $t=\infty$ and the inversion coefficient $K=2$. In the case of a narrow spectrum, the value of the inversion coefficient depends on the homogeneity of the radio frequency field, which is a constant, depending on the probe coil parameters. Experimentally estimated value of the inversion coefficient in the middle of the spectrum is 0.95 in our experiments on the AM-500 NMR spectrometer.

Broad spectrum is typical for ^{13}C-NMR spectroscopy and the spectrum offset $\Delta\omega$ should be accounted for in calculating the inversion. In the case of the usual single inverting π pulse of 20 μs duration, the macroscopic vector of magnetization reaches the x-y plane instead of inverting at 12.5 kHz offset from the middle of the spectrum. The use of a composite pulse sequence for inversion makes this problem less serious. It is evident from Fig. 1 that inversion is a smooth function of the offset as has also been predicted theoretically [5]. In the case of a composite π-pulse, the dependence of inversion coefficient on spectral offset $K(\Delta\omega)$ is well approximated with a parabola. With an independent estimation of the $K(\Delta\omega)$ we can decrease the number of parameters to be estimated

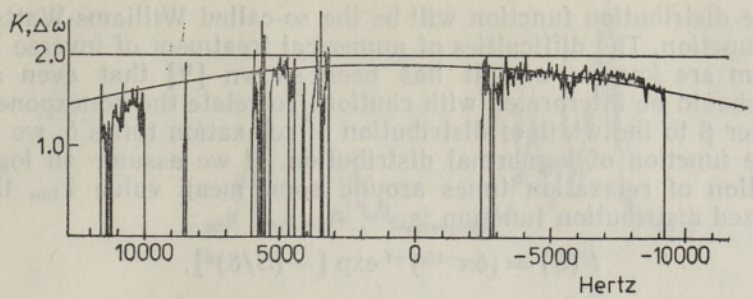

Fig. 1. Plot of the inversion coefficient $K(\Delta\omega)$ vs. spectral offset $\Delta\omega$ for the composite π-pulse used for solution spectroscopy. Straight line depicts ideal inversion and parabola is the approximation used in this work.

and enhance the precision of the calculated relaxation times. The equation used in our calculations is

$$I(t) = I_\infty[1 - 2K(\Delta\omega)\exp(-t/T_1)], \tag{3}$$

where

$$K(\Delta\omega) = 0.95 - 0.2(\Delta\omega/\Delta\omega_{max})^2 \tag{4}$$

and $\Delta\omega_{max}$ is the half-width of the spectrum.

The relaxation behaviour of macromolecules in solution is usually treated as exponential without experimental verification. The main reason for this is insufficient sensitivity of ^{13}C-NMR spectroscopy even at the high concentrations used.

In many different types of relaxation experiments, as in dielectric relaxation, photon correlation spectroscopy and mechanical relaxation, experimental data are analyzed with empirical fitting functions to deal with nonexponentiality. For the IRFT type experiment an analog of the so-called Williams-Watts function [6, 7] may be used

$$I_{ww}(t) = \exp[-(t/t_{ww})^{\beta_{ww}}]. \tag{5}$$

The parameter β_{ww} may be interpreted as a measure of the nonexponential character of the relaxation function and it determines the width of the distribution function (when β_{ww} becomes smaller, the distribution becomes broader).

To characterize the exponentiality of relaxation we introduce a parameter of exponentiality β into the formula describing the IRFT experiment

$$I(t) = A[1 - B\exp(-t/T_1)^\beta]. \tag{6}$$

If $\beta = 1$, this equation reverts to the usual formula describing the IRFT experiment. Calculations of the relaxation time constant T_1 and β from the 3-parameter Eq. (6) were done using the simplex algorithm [8, 9].

There are other possibilities to account for the nonexponentiality, such as using more exponents in the analysis. The experimentally achievable S/N ratio and a limited number of t values, however, do not permit this type of analysis. It has been shown [10] that several sums of exponentials may be consistent with the same set of data, and the question of uniqueness of representation is left open. The method used here to treat the nonexponentiality is computationally efficient and provides a direct relation with the distribution width of relaxation times if an appropriate distribution function is used.

The parameter of nonexponentiality is related to the distribution function of relaxation times through inverse Laplace transformation. If nonexponentiality is considered as arising from a superposition of exponen-

tials, the distribution function will be the so-called Williams-Watts distribution function. The difficulties of numerical treatment of inverse Laplace transform are known, and it has been shown [11] that even analytic results should be interpreted with caution. To relate the nonexponentiality parameter β to the width of distribution of relaxation times δ, we proceed from the function of lognormal distribution. If we assume an lognormal distribution of relaxation times around some mean value T_{1m}, then the normalized distribution function is

$$F(S) = (\delta\pi^{-1/2})^{-1} \exp\left[-(S/\delta)^2\right], \tag{7}$$

where

$$S = \ln\left(T_1/T_{1m}\right). \tag{8}$$

The quantity δ measures the half-width of the distribution at the point where $F(S)$ falls to e^{-1} of its maximum value, $F(0)$.

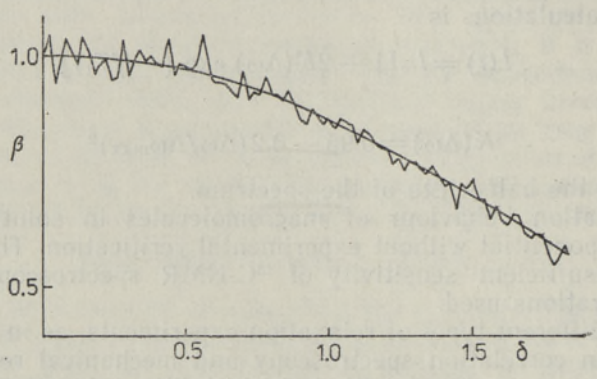

Fig. 2. Plot of the relaxation exponentiality β vs. width of the artificial data with lognormal distribution of width δ. Smooth line corresponds to error-free artificial data and the oscillating line contains 5% random error in data.

For computer-generated artificial IRFT experimental data, the dependence of β upon distribution width of relaxation times δ is represented in Fig. 2. In the range of $\delta = 0$ to 2.0 we found that values from two tau sets (used for liquid and powder experiments respectively) show very similar behavior. Adding random noise to simulated IRFT data causes no systematic errors in estimated β if $N/S < 5\%$.

Results and discussion

Fig. 3 shows a comparison of solid state and solution ^{13}C-NMR spectra of native carbonic anhydrase *B*. Both spectra have the same characteristic resonance regions: carbonyls at the low field side of the spectra, aromatic carbons at 110—140 ppm, α-carbons at 50—60 ppm, and aliphatic carbons at the high field.

Resonances in the powder spectra are broader, which is mainly a consequence of conformational inhomogeneity and the anisotropy of diamagnetic susceptibility in the amorphous sample. Slow overall tumbling with the rotational correlation time τ_R of 40 ns [12] broadens the lines and causes overlaps in the solution spectrum. The lines are still broader in the solid state powder spectrum. Spinning sidebands of carbonyl resonances are also present due to the large chemical shift anisotropy of the carbonyls ($CSA_{C=O} \simeq 200$ ppm).

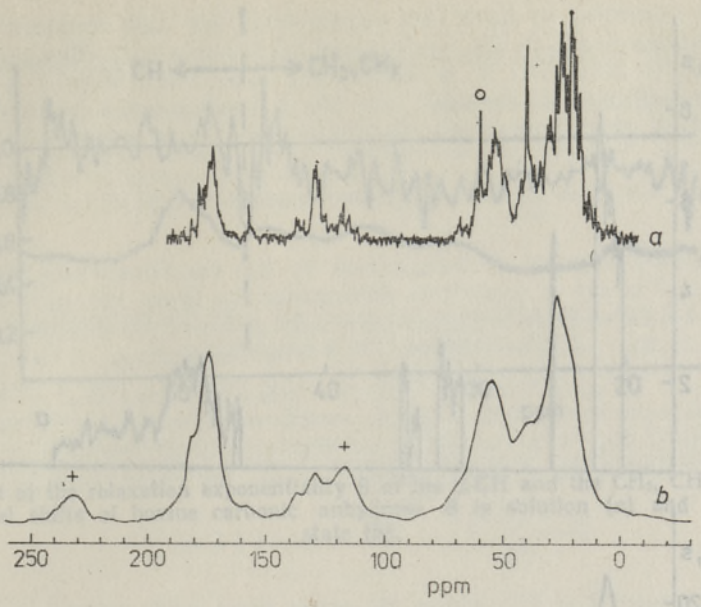

Fig. 3. ^{13}C-NMR spectra of bovine carbonic anhydrase *B* in solution at 11.7 Tesla (*a*) and in the solid state at 4.7 Tesla (*b*). + are the spinning sidebands of the carbonyls, O is TRIS buffer. Chemical shift is referenced to external TMS.

More obvious than in the usual NMR signal intensity spectra are the differences between the two states studied in the relaxation spectra. There is a substantial difference between the two relaxation spectra shown in Fig. 4, because in the case of solution the T_1 represents a true time constant of exponential relaxation, however for the powder sample the calculated value is the effective time constant of a multiexponential relaxation process.

The order of magnitude of the relaxation times in liquid state is determined by the overall rotation of the enzyme molecule. Differences between various parts in the relaxation spectrum are mainly caused by the number of directly connected protons. The variance inside each region is caused by internal dynamics which is an effective source of relaxation. The scattering in relaxation spectra gives also an estimate of error. The relaxation of powder sample is a manifestation of segmental dynamics. The most flexible part in the molecule are methyl groups with a relaxation time of 0.5 s.

In a protein molecule with many different carbon sites and degrees of freedom, many correlation times are needed, since we are dealing with an inhomogeneous system with a variety of microenvironments. Two approaches to describing the nonexponential relaxation behavior may be taken [11]. The first proposes that the relaxation behavior at the molecular level is nonexponential. The second approach interprets the nonexponential relaxation behavior in terms of a superposition of various exponentially relaxing processes, which then leads to the distribution of relaxation times. The assumption of a distribution of relaxation times, each characterizing an exponential relaxation function, seems physically more appropriate than the use of a single nonexponential relaxation function. If exponentiality $\beta<1$, then it may be caused by a nonexponential relaxation process or may be a consequence of a sum of exponents. When $\beta=1$, there is no distribution of relaxation times and the relaxation process should be described by a single time constant.

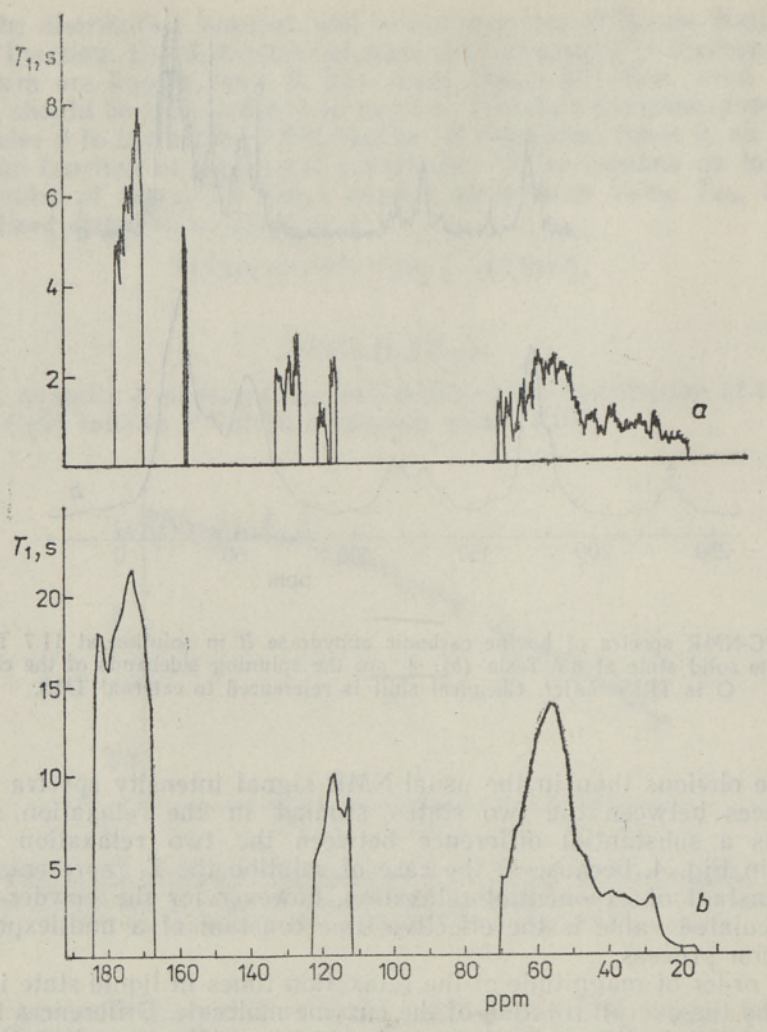

Fig. 4. Relaxation spectra (plot of relaxation times T_1 vs. chemical shifts) of bovine carbonic anhydrase *B* in solution (*a*) and in the solid state (*b*).

In the exponentiality spectra shown in Fig. 5, where the intensity is proportional to the relaxation exponentiality parameter β, a clear difference is obvious between the two states investigated. In the case of a powder sample, a nonexponential relaxation is found. In the exponentiality spectrum of the powder sample, a nearly constant value $\beta \simeq 0.6$ is characteristic for all spectral regions. In the liquid state the relaxation of α-carbons is characterized by a value of $\beta \simeq 1$.

Carbonic anhydrase is composed of 263 amino acid residues. The α-carbon resonances lie in the range of about 10 ppm (1250 Hz at 11.7T). If we take the typical width of a single resonance in solution equal to 30 Hz, and distribute the 263 resonances uniformly over this region, we can see that in every data point at least 6 nearby lines contribute at a level higher than half intensity. Due to overlap of resonances there exists a possibility of multiexponential relaxation if there are differences in the microdynamics of the various parts of backbone. There will be a sum of exponential processes and the exponentiality parameter will differ from one. In the case of carbonic anhydrase there is no evidence for nonexpo-

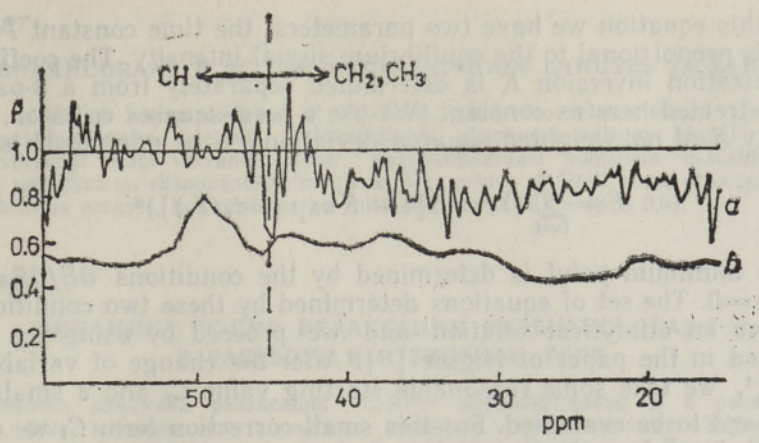

Fig. 5. Plot of the relaxation exponentiality β of the α-CH and the CH_2, CH_3 region vs. the chemical shifts of bovine carbonic anhydrase *B* in solution (*a*) and in the solid state (*b*).

nential relaxation and we may conclude that the relaxation times are rather uniform. Relaxation processes of aliphatic carbons feature a smaller value of exponentiality ($\beta=0.7$ to 0.9), which may be the consequence of more heterogeneous local motions than is characteristic for the α-carbons.

Conclusions

^{13}C-NMR spin-lattice relaxation behavior of carbonic anhydrase *B* was studied. Relaxation of all α-carbons in the enzyme molecule in aqueous solution is found to be exponential ($\beta\simeq 1$).

Relaxation of CH_3 and CH_2 groups in solution is governed by highly heterogeneous motions of the side chains, which leads to moderate nonexponentiality ($\beta\simeq 0.8$). Severely nonexponential relaxation process ($\beta\simeq 0.6$) is found to be characteristic for the powder sample. By analyzing the time course of magnetization recovery after inversion in terms of β, a clearly different relaxation behavior in the solid and liquid states is established.

In native carbonic anhydrase *B* under physiological conditions in solution, the α-CH groups participate in identical microdynamics. There is only a very narrow distribution of relaxation times, caused by minor local heterogeneity in conformational structures or dynamics, but the deviations from ideal exponentiality do not exceed 5%.

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APPENDIX

We have *N* experimental data points at delay times t_i with the corresponding intensities $Y_i=Y(t_i)$ which are described by the 2-parameter equation:

$$I(t)=B[1-K\exp(-t/T_1)].$$

In this equation we have two parameters: the time constant T_1 and B, which is proportional to the equilibrium signal intensity. The coefficient of magnetization inversion K is determined separately from a 3-parameter fit and treated here as constant. We use a least-squares criterion, and the quantity E of nonweighted squared deviations to be minimized is:

$$E=\sum_{i=1}^{N}(Y_i-B[1-K\exp(-t_i/T_1)])^2.$$

The minimum point is determined by the conditions $\partial E/\partial B=0$ and $\partial E/\partial T_1=0$. The set of equations determined by these two conditions does not have an analytical solution and we proceed by using the method described in the paper of Higbie [13]. After the change of variables $C=-T_1^{-1}$, we take some reasonable starting value C_0 and a small correction $C_1 \ll 1$ to be evaluated. For this small correction term C_1 we use only a linear term from Taylor series of the exponent function $\exp(C_1 t) \simeq 1+C_1 t$. Introducing symbol $e_i \equiv \exp(t_i C_0)$, the error term has the form

$$E=\sum_{i=1}^{N}(Y_i-B[1-Ke_i(1+C_1 t_i)])^2.$$

The substitution $B_1=BC_1$ gives the error term with two linearly dependent variables in the final form

$$E=\sum_{i=1}^{N}(Y_i-B(1-Ke_i)-B_1 Ke_i t_i)^2.$$

The minimum is now determined by two partial differentials $\partial E/\partial B=0$ and $\partial E/\partial B_1=0$, and the set of linear equations is:

$$B\{A_{1,i}^2\}+B_1\{A_{1,i}A_{2,i}\}=\{Y_i A_{1,i}\},$$
$$B\{A_{1,i}A_{2,i}\}+B_1\{A_{2,i}^2\}=\{Y_i A_{2,i}\},$$

where $A_{1,i}=1-Ke_i$ and $A_{2,i}=Ke_i t_i$, and $\{\}$ denotes summation over i. Solving this set of equations and substituting back variables, we get value for B and a better estimate for T_1. Starting from some reasonable initial value $(0.5<T_{1,\text{initial}}/T_{1,\text{final}}<2)$, less than 5 iterations converge to better than 0.1% precision for the fitting parameters T_1 and B.

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KARBOANHÜDRAAS *B* ^{13}C-TMR RELAKSATSIOON LAHUSES JA TAHKISES

On mõõdetud karboanhüdraas *B* ^{13}C-TMR spinn-võre relaksatsiooni ajalist kulgu inversiooni taastumise meetodil. Relaksatsiooni eksponentsiaalsust hinnati Williams-Wattsi võrrandi tüüpi võrrandi alusel. Karboanhüdraasi põhiahela α-süsinike relaksatsioon vedelikus on eksponentsiaalne ($\beta \simeq 1$) ja erineb oluliselt sama aine relaksatsioonist pulbrilises proovis, kus protsess on mitteeksponentsiaalne ($\beta \simeq 0{,}6$).

Ю. ЯРВЕТ

ДИНАМИКА ^{13}С-ЯМР РЕЛАКСАЦИИ КАРБОАНГИДРАЗЫ *B* В РАСТВОРЕ И В ТВЕРДОМ ТЕЛЕ

Изучалась динамика релаксации ^{13}С-ЯМР карбоангидразы *B* в растворе и в твердой фазе методом восстановления инверсии намагниченности. Экспоненциальность релаксации оценивали по аналогу уравнения Виллиамса—Ватта. Релаксация α-углеродов полипептидного остова в растворе является экспоненциальной ($\beta \simeq 1$) и отличается от релаксации в порошковом образце ($\beta \simeq 0.6$).