Thermodynamic and Magnetic Resonance Studies on the Hydration of Polymers: II. Protein-Water Interactions in Powdered Ribonuclease

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ESR studies on spin-labeled amorphous RNase A as a function of varying concentrations of sorbed H_2O and D_2O will be presented. A relaxation analysis of saturation transfer (ST-)ESR spectra of $^{14}N(^1H)$ nitroxide spin-label molecules essentially fixed at amino acid residue His-105 will be given.

A characteristic correlation has been observed between the microdynamic behavior – expressed by the rotational correlation times of the paramagnetic label – and the macroscopic thermodynamic entropy for the sorption process of H_2O and D_2O at RNase. This correlation is particularly pronounced at low water concentrations, *viz.*, $n_{H2O}/n_{protein} \leq 100$. A significant difference in this concentration range exists between the two systems "RNase-H_2O" and "RNase-D_2O", which is manifested not only by the thermodynamic data but also by the microdynamic behavior extracted from the corresponding non-linear ESR absorption line shapes.

Introduction

The hydration of proteins is a phenomenon of considerable biological importance and physicochemical interest. An understanding of this hydration requires information about the nature and strength of water-protein interactions; the structural arrangement, if any, of the hydration of water; the mobility of the water molecules within the structural arrangement; and the effects of hydration on the structure, the dynamics, and the enzymatic activity of the protein itself [1].

In the present investigation an attempt is made to adduce such information from the results of ESR relaxation and thermodynamic measurements of (spin labeled) bovine pancreatic ribonuclease A (RNase A) with varying degree of hydration. The amount of sorbed water (H_2O, D_2O) was controlled accurately by equilibration with water at known activities [2]. RNase was labeled with N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide (BSL) at the position of the N-3 atom of histidine-105 [3]. In the following discussion, the labeled derivative will be abbreviated as "BSL-RNase". Although the BSL reporter group is fixed near the active center of RNase, and therefore gives information on conformational changes from this molecular region, its influence on the enzymatic activity is negligible small [4].

From nitroxide radical spin-label probes much useful information can be obtained from the effects of motion of the probe on the ESR spectrum [5]. Spectral lineshapes show maximum sensitivity to motion when the rotational correlation time is of the same order of magnitude as the inverse of the anisotropy of the magnetic interactions, typically 10^{-9} s. Therefore, with conventional (linear) ESR spin label experiments, motional effects are evident in the lineshape of the spectra, which are characterized by correlation times of the reporter group in the range between 10^{-11} s and 10^{-7} s. For powdered protein samples (with varying water activities) as they were investigated in the present study, however, the mobility of the spin probe is drastically diminished resulting in correlation times between 10^{-3} s and 10^{-6} s; a dynamical range, in which the conventionally detected ESR spectra are insensitive to molecular random motions. In order to extract dynamic data from this "very slow motional" region by an ESR

Abbreviations: RNase, ribonuclease A; BSL, N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide.

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lineshape analysis, non-linear techniques such as saturation transfer (ST-)ESR spectroscopy are appropriate [6].

This technique has been applied in the present study for an analysis of ESR spectra of "BSL-RNase" at varying degrees of hydration. At low water concentrations ($h < 20$) the extracted dynamical data correlate with corresponding thermodynamic data, such as the entropy or enthalpy of the sorption process.

Experimental Section

Materials

Ribonuclease A from bovine pancreas (EC 3.1.27.5) was obtained from Fluka, and the BSL nitroxide label from Aldrich. RNase was labeled as described in the literature [3] without the final ion exchange chromatography. The spin concentration was determined by ESR using a reference sample of known BSL concentration. In order to circumvent line broadening by electron spin-spin interaction, the spin-labeled RNase was diluted with its unlabeled analogue by a ratio of 1:40.

Sample preparation

ESR samples were prepared by equilibrating lyophilized RNase at 25 °C in U-shaped quartz tubes on a high vacuum line, connected with a flask containing a mixture of (thoroughly degassed) sulfuric acid and water of known composition (*cf.* ref. [7]). The vapour pressures, p, of these mixtures at different temperatures were taken from the literature [8]. Parallel to the sample equilibration, the sorption isotherms of RNase were measured in a thermobalance of the type described elsewhere [9]. Equilibrium was assumed until a state had been achieved, where no further changes of the weight (within the experimental error interval of $\pm 2 \times 10^{-7}$ g) over a period of at least two days were observable. For the ESR measurements the sample tubes were sealed. Sorption temperature was controlled to be better than 0.5 K over a period of several weeks.

ESR measurements

All ESR spectra were recorded at 9.5 GHz with a VARIAN-E 209 spectrometer equipped with a E 207 modulation and detection unit. Sample temperatures were controlled by a stream of precooled nitrogen using the VARIAN V-6040 variable temperature assembly. ST-ESR signals were registered at a microwave power, where the maximum of the corresponding ESR signal did occur. At this power level an optimum between weak saturation of the spin ensemble and reproducibility of the spectra can be achieved. The "90°-out-of-phase" calibration of the detector system was performed by the "self-null-method" as proposed by Thomas *et al.* [10].

In general there are eight displays ($U_{1,2}$, $U'_{1,2}$, $V_{1,2}$, $V'_{1,2}$) which have to be considered in an ST-ESR experiment, where U refers to the dispersion mode, V stands for the absorption mode and the prime on either U or V means that the "out-of-phase" response is detected. The subscript corresponds to the particular harmonic of response. In the present investigation, ST-ESR experiments have been observed only in the V'_2 mode, *i.e.*, the second harmonic out-of-phase absorption.

Computer simulations

The g-tensor and the hyperfine tensor A were determined by a least-square-fit method [11]. The ESR spectra in the "slow motional" region were simulated by the model of Freed *et al.* [12]. All calculations and spectra simulations were executed on a CYBER 175 computer.

Results

Sorption isotherms

The amount of sorbed water (H_2O or D_2O) is given by the ratio of weight h = g(water)/g(dry protein). The sorption isotherms at 298 K are presented in Fig. 1 through h as a function of the water vapour activity $a_w = p/p_o$, where p_o is the saturation vapour pressure given by the sulfuric acid-water mixture at a particular temperature. The different isotherms marked by roman numbers correspond to various sorption cycles. Curve I refers to the first sorption process on lyophilized RNase; whereas curve II represents the second and all following sorption experiments following an exhaustive dehydration from the previously sorbed water on a high vacuum line. The desorption branches which are given by curve III are identical for both sorption processes I and II. Sorption isotherms with D_2O were obtained from lyophilized RNase which was dissolved in D_2O, so that all exchangeable protons were replaced by deuterium.

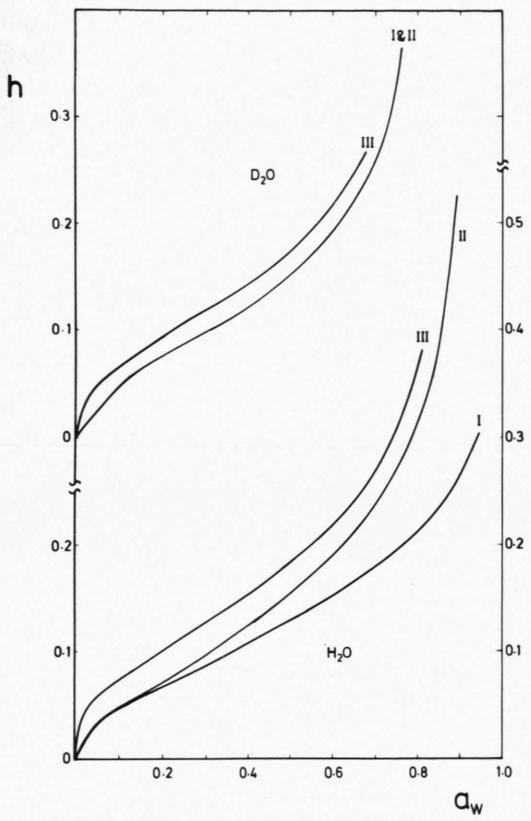

Fig. 1. Sorption isotherms of H_2O and D_2O on RNase at 298 K. Curve I: first sorption cycle; curve II: second and following sorption cycles; curve III: desorption.

The sorption temperature was 298 K and 277 K, its accuracy was better than 0.5 K.

ESR experiments

For a quantitative analysis of ST-ESR spectra, a parametrization technique similar to the one described by Thomas *et al.* [10] has been applied. The parameters, which are most sensitive to dynamical processes in the V'_2 detection mode, are given by the ratios (H″/H, L″/L, C′/C) as depicted in Fig. 2. For the procedure used in the present study, the components of the *g*-tensor and the hyperfine coupling tensor *A* of powdered "BSL-RNase" as well as of "BSL-RNase" in glycerol have to be known for reasons which will be discussed in more detail below (next section).

The lineshape of "BSL-RNase" dissolved in glycerol resembles that of a powder spectrum and therefore can be simulated by the method of least-square-fit as described in ref. [11]. The results are collected in Table I together with corresponding data of powdered "BSL-RNase". Within experimental error a fairly good agreement exists between both sets of data. This will be of importance for the determination of the correlation times from spectra parameters described in the following section.

Table I. *g*-Tensor and hyperfine tensor *A*. a) "BSL-RNase" in glycerol; b) "BSL-RNase" with 0% sorbed water.

(a)		
$g_{pp} = 2.00583$		$A_{pp} = (0.482 \pm 0.13)$ mT
$g_{qq} = 2.00666$	± 0.00055	$A_{qq} = (0.809 \pm 0.06)$ mT
$g_{rr} = 2.00217$		$A_{rr} = (3.245 \pm 0.02)$ mT
$1/3 * \mathrm{Tr}(g) = 2.0049$		$1/3 * \mathrm{Tr}(A) = 1.512$ mT
(b)		
$g_{pp} = 2.00607$		$A_{pp} = (0.466 \pm 0.07)$ mT
$g_{qq} = 2.00757$	± 0.00055	$A_{qq} = (0.639 \pm 0.04)$ mT
$g_{rr} = 2.00210$		$A_{rr} = (3.296 \pm 0.01)$ mT
$1/3 * \mathrm{Tr}(g) = 2.0052$		$1/3 * \mathrm{Tr}(A) = 1.467$ mT

Discussion

Thermodynamic data

In dealing with sorption on polymers, it is reasonable to define the thermodynamic standard state (with a zero free enthalpy) for the swollen polymer under its saturation pressure, p_o [13–15]. The partial molar free enthalpy of water is therefore given as

$$\Delta\bar{G} = \frac{\delta\, \Delta G(T,p,n)}{\delta\, n} = RT \ln p/p_o = RT \ln a_w. \qquad (1)$$

From $\Delta\bar{G}$, the partial molar enthalpy $\Delta\bar{H}$ and entropy $\Delta\bar{S}$ of sorption can be calculated, *viz.*,

$$\Delta\bar{H} = \frac{\delta\, \Delta H(T,p,n)}{\delta\, n} = \frac{R\, T_1 T_2}{T_1 - T_2} \ln (a_{w1}/a_{w2}) \qquad (2a)$$

$$\Delta\bar{S} = \frac{\delta\, \Delta S(T,p,n)}{\delta\, n} = \frac{\Delta\bar{H} - \Delta\bar{G}}{T} = \frac{\Delta\bar{G}_1 - \Delta\bar{G}_2}{T_1 - T_2}. \qquad (2b)$$

Fig. 3 shows $\Delta\bar{S}$ as function of h, the amount of sorbed water for the different sorption cycles on RNase. For H_2O the partial molar entropy $\Delta\bar{S}$ is characterized by several distinct maxima and minima which are developed most at low water concentration. Table II collects some molecular data of

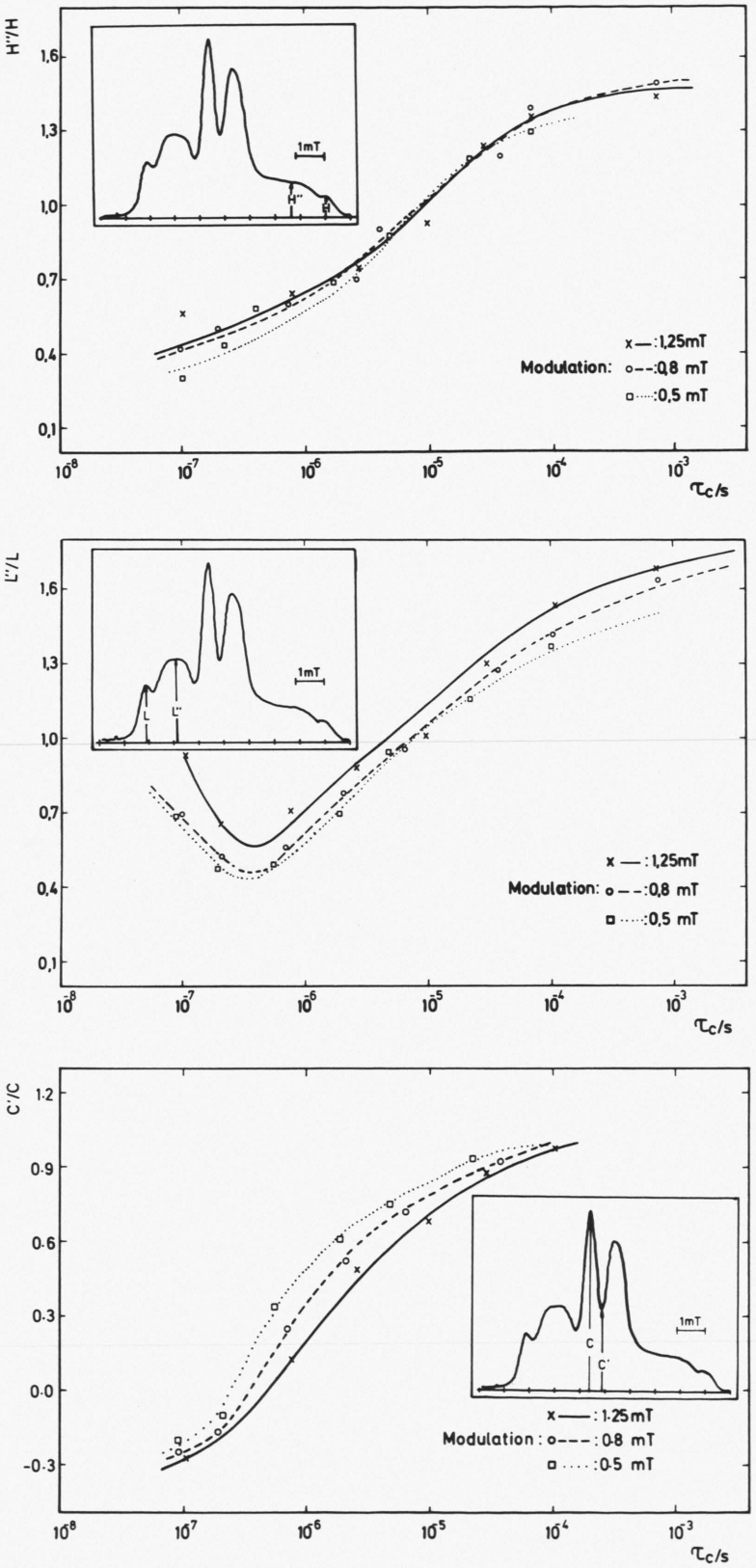

Fig. 2. Definition of the spectrum parameter H''/H, L''/L and C'/C and their dependence on the correlation time τ_c at varying modulation field strengths.

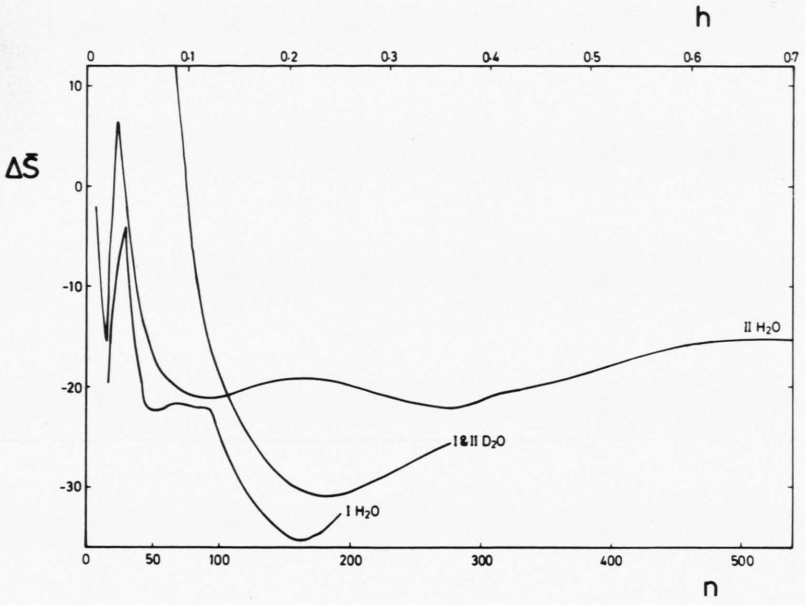

Fig. 3. Partial molar entropy of sorption $\Delta\bar{S}$ as function *versus* the amount of sorbed water: h = gram{H_2O}/gram{protein}; $n = n_{H2O}/n_{protein}$.

RNase. These data show, that – within the experimental error interval of ±4 sorbed water molecules per protein molecule – a correlation exits between the number of charged and polar groups and the number of sorbed water molecules at the extremal properties of $\Delta\bar{S}$ and $\Delta\bar{H}$. Position #1 of Table II gives the number of isolated charged polar groups (at pH 6–7, *cf.* ref. [16]) which corresponds to the number of water molecules at the minimum of $\Delta\bar{S}$ for the 2nd and all following sorption processes. Although the experimental data points in the very low concentration region are not sufficient in the present investigation in order to give a more quantitative analysis, it is interesting to note, that corresponding

Table II. Binding sites of RNase for water sorption.

Position	Number of binding sites	Description of binding sites		Water concentration $n = n_{H2O}/n_{protein}$
#1	18	18	isolated charged groups	15
#2	10	18 + 5	five neighboured ion-pairs	23
#3	59	18 + 10 + 59	10 ion-pairs 59 polar groups	90
#4	164	175 −11	exchangeable H-bonds intermolecular H-bonds	160
#5	272	379 −107	hydrophilic groups intra- and intermol. bonds	270

changes of the specific heat capacity c_p ($= T \cdot \delta S/\delta T$) have also been observed for lysozyme at low concentrations ($h \leq 10$) [17].

Considering the five pairs of oppositely charged polar groups in RNase which are neighboured, a sorption model may be envisaged, where two such groups on the average share one water molecule. Such a model rationalizes the maximum value of $\Delta\bar{S}$ for the sorption and desorption (not shown in Fig. 3) processes of H_2O observed on RNase. Between the total of 59 uncharged and 28 charged polar groups and the minimum of $\Delta\bar{S}$ at $n \approx 90$ again a correlation seems to exist (#3 in Table II). Position #4 in Table II gives the total amount of 175 exchangeable protons [18] diminished by the number of 11 possible intermolecular hydrogen bonds [19] resulting in 164 distinguished positions; a number which correlates with the position of the broad entropy maximum at $n \approx 160$. If, on the other hand, all of the 379 hydrophilic groups in RNase are considered [20], and if their number is reduced by the 107 intra- and intermolecular H-bonded groups [19], the resulting number of 272 hydrophilic groups again corresponds to an extreme value of $\Delta\bar{S}$ (#5 in Table II).

The maximum value of $\Delta\bar{S}$ (at $n \approx 30$) for the first sorption cycle of H_2O on RNase corresponds to the total number of 28 charged groups (position #1 and #2 in Table II) if it is assumed that no ion-pairs have been developed at the beginning of the sorption process on the lyophilized protein; while the position of the broad minimum for the first cycle of H_2O at $n \approx 160$ again may be correlated with the values of position #4 in Table II. The minimum of $\Delta\bar{S}$ itself may be rationalized by the idea that intermolecular hydrogen bonds are created during the first sorption cycle of H_2O on RNase. Such a conception would be in good agreement with the observed macroscopic changes of the state of aggregation, namely from a loose (electrostatically charged) powder to a spongy material, the remaining state of the protein during all following sorption cycles. Such a process partially explains the hysteresis between the first and all following sorption cycles for H_2O on RNase.

For D_2O on the other hand, neither a hysteresis has been observed between the differing sorption cycles nor macroscopic changes of the state of aggregation seem to occur. The position of the minimum value of $\Delta\bar{S}$ at $n \approx 180$, a slightly higher water concentration if compared to H_2O, indicates that no such intermolecular hydrogen bonds are developed for the "RNase-D_2O" system, *i.e.*, the possible 11 groups are obviously occupied by sorbed D_2O molecules.

Although the results collected in Table II suggest a strong correlation between the number of hydrophilic groups in RNase and the sorption process represented by the thermodynamic properties of Eqns. (1, 2), the development of a simple sorption model describing not only the isotherms but also the thermodynamic quantities $\Delta\bar{G}$, $\Delta\bar{S}$, and $\Delta\bar{H}$ in an adequate way is strongly hampered by conformational changes which occur within the protein matrix during the sorption process. It is quite obvious that voids are created on drying a protein which give rise to high unsatisfied intermolecular forces, that cause substantial refolding of the polypeptide chains in order to reduce the size of these voids. If, for example, the surface energy is assumed to be of the order of 5×10^{-6} J cm^{-2} – a reasonable value for a protein – then a spherical void 1.0 nm in diameter would have a surface energy of about 100 kJ per mole of voids, *i.e.*, the dried protein crystals without conformation changes of the polypeptide chains would be characterized by an energy, which is at least several tens of kilojoules higher than for its analogue with less unoccupied voids. The hysteresis observed for the sorption and desorption isotherms as well as the difference of the first and all following sorption cycles (*cf.* Fig. 1) have to be considered as a reflection of such processes of conformational rearrangements within the polypeptide matrices. For an interpretation of the measured enthalpy and entropy associated with the sorption process, this may be expressed by a simple model, *viz.*:

$$(\text{protein})_A + n\text{W}_1 \rightleftharpoons (\text{protein})_B + n\text{W}_2 \qquad (3)$$

where $n\text{W}_1$ is the water species (vapour) converted to $n\text{W}_2$ (sorbed) when the protein in its (conformational) state A is converted to the hydrated form B with

$$\begin{aligned} \Delta\bar{H} &= \Delta\bar{H}_{A-B} + \Delta\bar{H}_{nW1-nW2} \\ \Delta\bar{S} &= \Delta\bar{S}_{A-B} + \Delta\bar{S}_{nW1-nW2}. \end{aligned} \qquad (4)$$

In Eqn. (4) the terms $\Delta\bar{H}_{A-B}$ and $\Delta\bar{S}_{A-B}$ reflect all conformational changes within the protein matrix during the sorption or desorption process, and these terms certainly cannot be neglected. In all models for the description of the sorption isotherms, however, only the strength and number of different binding sides have been considered without taking into ac-

count possible conformation changes of the protein matrix itself.

In the present investigation the model by Hailwood and Horrobin [21] in its modification by D'Arcy and Watt [22] was used for a simulation of the experimentally determined isotherms. Although good agreement between the experimental data points and the calculated isotherms exists, the adjustable parameters of the model equation differ not only for the various sorption cycles but also for different temperatures. This again demonstrates that conformation changes cannot be neglected, neither for an interpretation of the isotherms, nor for a quantitative understanding of the thermodynamic parameters such as, *e.g.*, the entropy of sorption. This argument also holds for the results observed in case of the D_2O sorption process on RNase (*cf.* Fig. 3), where no differences between the first and all following sorption cycles have been observed and where $\Delta\bar{S}$ as a function of h, the water concentration only shows one broad minimum at $n \approx 170$.

ESR experiments

The determination of rotational correlation times from saturation transfer spectroscopy can either be performed by i) theoretical computer simulations, or by ii) a parametrization technique on model systems [10]. Besides the need of a relatively high computer capacity, the dependency on several unknown parameters unfavors a theoretical computer simulation. In the present investigation therefore we preferred a parametrization technique, similar to the one described in ref. [13]. First, ESR spectra were simulated in the so-called "slow motional region" of BSL-RNase dissolved in glycerol at varying temperatures. For these simulations the model of Freed *et al.* [12] was used. The parameters which are necessary for such a spectroscopic analysis are the g-tensor and the hyperfine tensor A, which are collected in Table I. Furthermore an isotropic rotational diffusion model was assumed for the stochastic motion of the spin label in the protein matrix.

The ESR spectrum of "BSL-RNase" in glycerol is composed by two components, which have been interpreted as a result of different mobility caused by two label configurations in RNase [3]. For both, a reversible temperature dependence between 303 K $\leq T \leq$ 353 K has been registered. They were separated by a superposition of two ESR spectra in the "slow motional region". Details on the separation will be reported elsewhere [23]. From the correlation times of the slowly tumbling component (*cf.* ref. [23]) an Arrhenius plot was extracted and extrapolated in the region of "very slow motion" – corresponding to temperatures below 290 K. This is shown in Fig. 4 (solid line). These (extrapolated) values of τ_c represent the basis for a parametrization of the ST-ESR spectra. Fig. 2 depicts the calibration curves for the characteristic spectroscopic ratios L″/L, H″/H, and C′/C at different magnetic modulation field strengths. These results are very similar to

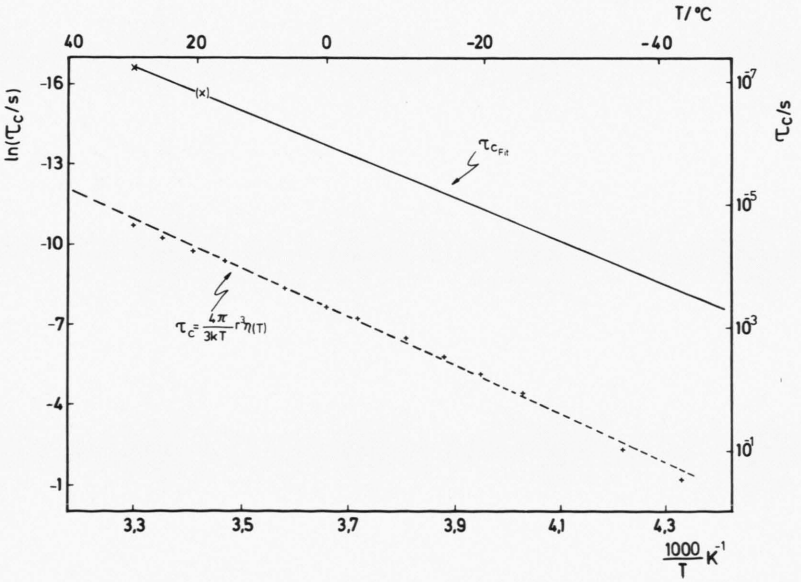

Fig. 4. Arrhenius plot of τ_c for "BSL-RNase" in glycerol. The solid line gives the extrapolated values for the temperature range below 293 K (see text). The broken line represents τ_c as calculated from Eqn. (5).

corresponding findings in ref. [10], obtained on maleimide-labeled oxyhemoglobin in glycerol-water mixtures. The dashed line in Fig. 4 gives the correlation times calculated from the macroscopic viscosity, η, of glycerol at varying temperatures for a sphere 6.6 nm in diameter as for RNase [18], using Debye's equation for the Brownian rotational diffusion, *viz.*,

$$\tau_c = \frac{4 \cdot \pi}{3 \cdot kT} \cdot r^3 \cdot \eta(T). \qquad (5)$$

The difference between the two curves in Fig. 4 reflects the possibility of an additional molecular mobility of the label in RNase, on one side, and possible shortcomings of the model (macroscopic viscosity, spherical shape, etc.) underlying the deviation of Eqn. (5), on the other side.

From the calibration curves in Fig. 2 the correlation times of "BSL-RNase" at varying degrees of hydration have been determined. The results are shown in Fig. 5. First it can be seen that for the sorption of H_2O the correlation times τ_c as a function of h, the water concentration, changes its slope drastically in the concentration range between $5 \leq h \leq 20$, a behavior which is also characteristic for the slope of $\Delta\bar{S}$ *vs.* h in this concentration range (*cf.* Fig. 2). This corroborates the assumption that conformational changes within the protein matrix determine the changes of $\Delta\bar{S}$ at low humidities. The different values of τ_c extracted from the ratios L″/L and C′/C (*cf.* Fig.5) can be rationalized by the possibility of different diffusion mechanisms of the spin marker molecule fixed in the protein matrix, as they occur for the protein dissolved in glycerol and the dried material at varying degrees of hydration. The influence of different diffusion models on parts of the ST-ESR spectrum has been studied [24], and it was shown that the dependency of L″/L on the diffusion mechanism is more pronounced than it is for the ratio C′/C reflecting spectral changes in the central part ($m_I = 0$) of the ST-ESR spectrum.

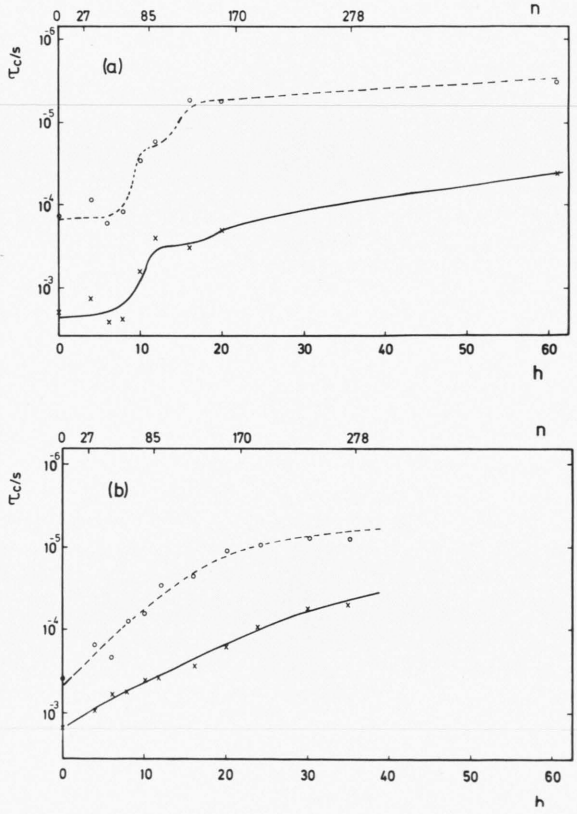

Fig. 5. Correlation times τ_c of "BSL-RNase" at varying degrees of hydration for H_2O (a) and D_2O (b). Dashed line: values of τ_c from L″/L; solid line: values of τ_c from C′/C.

Conclusions

In summary, one can state, that a correlation exists between the macroscopic observable thermodynamic quantities, such as the entropy of sorption and those physical parameters which reflect the microdynamical behavior of spin label molecules located near the active center of RNase. These correlated effects (at low water concentrations) have been attributed to confirmational changes within the protein matrix. Whether the conformation changes are localized at the active center or if they are distributed over the RNase molecule is subject of a forthcoming study on differently labeled RNase [23].

There, the dependency of the hyperfine coupling constant A of the spin label on the temperature and water concentration, which have not been reported in the present study, will be discussed in connection with possible different sequential steps of sorption for H_2O and D_2O. This analysis might give further insight in the differing sorption processes of H_2O and D_2O.

Acknowledgement

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[1] J. L. Finney, J. M. Goodfellow, and P. L. Poole, The Structure and Dynamics of Water in Globular Proteins, in: Structural Molecular Biology (D. B. Davies, W. Saenger, and S. S. Danyluk, eds.), p. 387, Plenum Press, New York 1982.
[2] E. von Goldammer, A. Müller, and B. E. Conway, Ber. Bunsenges. **78,** 35–42 (1974).
[3] R. R. Matheson, H. Dugas, and H. A. Scherarga, Biochem. Biophys. Res. Comm. **74,** 869–878 (1977).
[4] M. M. Gregory, E. W. Daniel, and R. G. Hiskey, Biochemistry **17,** 2025 (1978).
[5] L. J. Berliner (ed.), Spin Labeling, Theory and Applications, Academic Press, New York 1973.
[6] C. R. Dalton, B. H. Robinson, L. A. Dalton, and P. Coffey, in: Advances of Magnetic Resonance (J. S. Waugh, ed.), **Vol. VIII,** Academic Press, New York 1979.
[7] E. von Goldammer, B. E. Conway, D. H. Paskovich, and A. H. Reddoch, J. Polym. Sci. **11,** 2767 (1970).
[8] J. Timmermans, in: Physico Chem. Constants, **Vol. IV,** New York 1960; D'Ans-Lax, in: Taschenbuch für Chemiker und Physiker, Springer, Berlin 1967.
[9] W. B. A. Sharp and D. Mortimer, J. Sci. Instr. (J. Phys. E), Ser. 2, **1,** 843 (1968).
[10] D. D. Thomas, L. R. Dalton, and J. S. Hyde, J. Chem. Phys. **65,** 3006 (1976).
[11] H. J. Steinhoff, private communication.
[12] J. H. Freed, G. V. Bruno, and J. Polnascek, J. Phys. Chem. **75,** 3385 (1971).
[13] G. Dickel and J. W. Hartmann, Z. physik. Chem. NF **23,** 1 (1960).
[14] M. Dole and A. D. McLaren, J. Americ. Chem. Soc. **69,** 651 (1947).
[15] S. Davis and A. D. McLaren, J. Polymer. Sci. **3,** 16 (1948).
[16] A. L. Lehninger, in: Biochemistry, Worth Publ. 1970.
[17] P. H. Yang and J. A. Rupply, Biochemistry **18,** 2654 (1979).
[18] H. A. Scherarga, in: Protein Structure, Academic Press, New York 1961.
[19] C. H. Carlisle, R. A. Palmer, S. K. Muzumdar, B. A. Gorinsky, and D. G. R. Yeates, J. Mol. Biol. **85,** 1 (1974).
[20] G. Careri, E. Gratton, P. H. Yang, and J. A. Rupply, Nature **284,** 572 (1980).
[21] A. J. Hailwood and S. Horrobin, Trans. Far. Soc. **42B,** 84 (1946).
[22] R. L. D'Arcy and I. C. Watt, Trans. Far. Soc. **66,** 1236 (1970).
[23] E. Matuszak, E. v. Goldammer, and H. Wenzel, work in progress.
[24] V. Segre, J. Mag. Res. **41,** 432 (1980).