Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIII, No. 5

Probing Protein Structure and Dynamics by Tritium NMR

T. M. O'Connell[a], P. G. Williams[b]*, and J. T. Gerig[a]*

[a]Department of Chemistry
University of California
Santa Barbara, CA 93106

[b]The National Tritium Labeling Facility
Lawrence Berkeley Laboratory 75-123
University of California
Berkeley, CA 94720

SUMMARY

Tritium nmr spectroscopy of specifically tritiated tosylchymotrypsin has been used to examine the properties of the tosyl group in this protein. The unavoidable presence of several tritiated isotopomers complicates analysis of experiments and extensive computer simulations of relaxation behavior of tritiated species present were used in conjunction with models developed from crystallographic results to interpret the observations made. These analyses suggest that the tosyl group of tosylchymotrypsin at pH 4 is highly mobile in solution and occupies the location in the protein that is observed in the crystalline state only about 50% of the time.

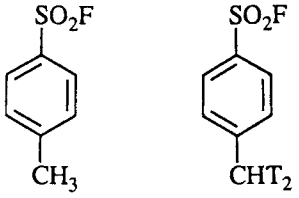
Keywords: *tritium nmr; tosylchymotrypsin; nmr reporter groups; protein*

INTRODUCTION

It is often possible to obtain many of the unique structural and dynamical insights that nmr can provide about proteins under conditions that are not favorable to multi-dimensional, multi-nuclear approaches by the use of extrinsic probes or "reporter" nuclei, in the same manner that nitroxides or other paramagnetic species are employed in esr experiments with biological systems. Some desirable features of a good reporter nucleus include a spin number of ½ so that lines are not broadened by quadrupolar relaxation, high sensitivity to detection, absence of background signals (either from the instrumentation used or spins intrinsic to the protein), ease of incorporation by synthesis into the system of interest, and absence of interactions with the reporter group that perturb the structure or dynamics that are to be explored. Tritium offers many of these *desiderata* and is especially attractive since tritium, after all, is hydrogen, and there should be no structural alterations upon its introduction into proteins.

0362–4803/93/050371–10$10.00

The serine protease α-chymotrypsin reacts stoichiometrically with *p*-toluenesulfonyl fluoride (**I**) to give an inactive protein with a tosyl group attached to the critical Ser-195 residue of the active site. The native enzyme and its tosyl derivative have been extensively studied by x-ray crystallography and it has been shown that in the solid state there is a cavity on the enzyme surface, often called the "tosyl pocket", into which the tosyl group fits (1). If this structure is strictly maintained in solution, the tosyl group would be held within the tosyl pocket and its aromatic ring unable to undergo rotation. In the present work the methyl protons of the tosyl group were replaced by tritons and examined by a variety of tritium nmr experiments. The results show that in solution at pH 4 tosylchymotrypsin does not completely maintain the orientation for the tosyl group observed in the solid state.



I **II**

RESULTS

Preparation of Tritium-labeled Tosyl Fluoride. Catalytic hydrogenolysis of the 4-dichloromethylbenzenesulfonyl fluoride was used to synthesize the methyl tritium-labeled tosyl fluoride. Although the starting dichloride was thought to be pure, the products of these reactions were mixtures of species with variable number of tritons. Consideration of proton and tritium nmr spectra of samples of **II** showed that they typically consisted of 5%, 43%, 46% and 7% of the CT_3, CHT_2, CH_2T, and CH_3 species, respectively. There was no detectable incorporation of tritium into the aromatic ring of the inactivator.

Tritium Spectra of Tosylchymotrypsin. Tritium spectra of enzyme modified with **II** exhibit broad lines due to the long correlation time of the protein and it was not possible to resolve signals for the various isotopic components. The linewidth for each species is of utility in helping to define the dynamics of the tosyl group and to obtain some estimate of these, we fit the observed lineshape to a sum of Lorentzian functions. In this process it was assumed that the isotopic composition of the tosyl groups attached to the enzyme was the same as that observed for the corresponding tosyl fluoride and that the chemical shifts, including isotope shifts, and coupling constants that were obtained from analysis of the spectra of the inhibitor molecule are transferable to the enzyme derivatives. It was assumed that the ^1H spectrum observed for the residual HOD in the solvent provided a reliable indicator of field inhomogeneity and the magnitude of spinning artifacts, both of which were taken into account.

For the enzyme modified with **II** the tritium lineshape was well-fit by assuming that the linewidths for the CH_2T, CHT_2, and CT_3 groups were 13, 14, and 15 Hz, respectively. The ordering of linewidths observed is that expected, since tritium is more effective in dipolar relaxation than protium, but the differences are within the reliability of the analysis.

Tritium Relaxation. The spin-lattice relaxation behavior of the composite line observed in these experiments was describable by a single exponential function. The apparent T_1 relaxation time is given in Table I.

Irradiation at the proton frequency of samples of the specifically tritium labeled enzymes produces an $^3H\{^1H\}$ NOE which is substantial and negative (Table I), consistent with dipolar interactions with neighboring protons being an important component of tritium relaxation. The same experiment with enzyme containing a tritiated tosyl group having the remaining tosyl protons replaced by deuterium shows a $^3H\{^1H\}$ NOE of nearly the same magnitude. Thus, proton-tritium dipole-dipole interactions must take place with spins attached to amino acids of the protein that are adjacent to the tosyl group.

The nature of these interactions was explored further by means of two-dimensional proton-tritium Overhauser experiments. Under the conditions of our experiments only a single set of NOE crosspeaks was observed for the composite tritium signal and the information contained in a 2D NOE spectrum can be represented by a "skyline" projection plot, in which crosspeaks appear at positions along the ^1H axis corresponding to the chemical shifts of protons which contribute to tritium-proton dipolar relaxation pathways. Figure 1 presents typical skyline plots from two-dimensional NOE experiments with enzyme modified with **II**. The strong features that appear in these spectra near 2.2 and 7.0 ppm at short mixing times must arise from interactions between the tritium nuclei and protons on the methyl group and aromatic ring of the tosyl group, since these features in the 2D results are greatly attenuated when the protons on the tosyl group are replaced by deuterium.

Spin diffusion is expected to be rapid in proteins as large as chymotrypsin (2) and we therefore examined the build-up rates of the $^3H\{^1H\}$ NOE crosspeaks. It is clear from these experiments that other protons with shifts between 2.5 and 4.6 ppm, in addition to the protons of the tosyl group, appear in the 2D NOE spectra because of direct interactions with the tritium nuclei of the tosyl methyl group. Using the signals from the protons of the aromatic ring as a standard, initial slopes of the build-up curves suggest that protein-bound protons with shifts of 2.9, 3.7, and 4.1 ppm are less than 0.3 nm away from the tritium nuclei.

A significant crosspeak was also observed at 4.6 ppm. This feature likely includes the effects of interactions of the tritium spins with residual protons in the D_2O solvent or protons which are solvent-exchangeable because increasing the amount of protium in the solvent increases the intensity of this peak. However, the 2D NOE experiments were carried out with

samples in which the solvent was >99% deuterated and intensity in the NOE data at this shift probably also represents an interaction with carbon-bound protons of the enzyme.

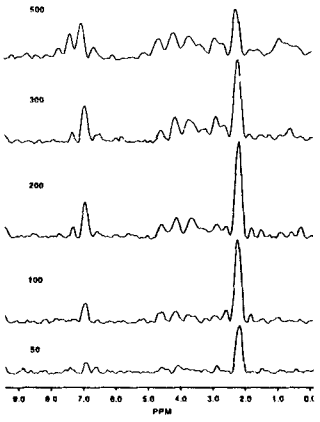


Figure 1. Skyline projections of two-dimensional 3H{1H} NOE experiments at 320 MHz with methyl tritium-labeled tosylchymotrypsin prepared from II. Mixing times (in ms) are indicated.

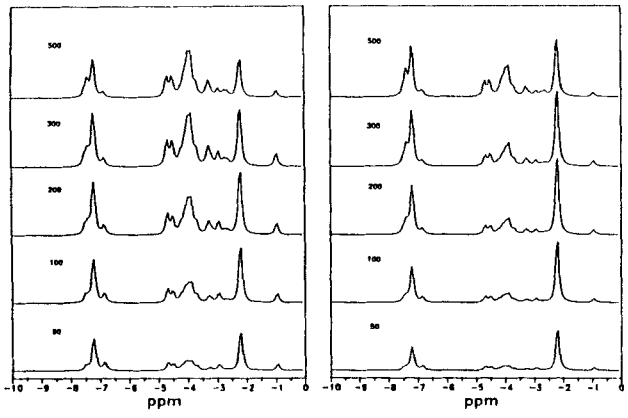


Figure 2. Computer simulations of two-dimensional 3H{1H} NOE experiments using the model described in the text. Mixing times (in ms) are indicated. The left panel presents computed skyline projections for proteins in which the tosyl group occupies the tosyl pocket 100% of the time, while the right panel gives results for when the tosyl group rapidly equilibrates between the "tosyl in" and "tosyl out" conformations, spending half of its time in each. Intensities as a function of mixing time for a given series are plotted on the same vertical scale but these scales vary from series to series. Protons from the solvent were not considered in the simulations.

Analysis of Data. Attempts to extract quantitative information from the tritium nmr spectroscopic observations made were complicated by the fact that these spectral responses are from a collection of isotopomers. To approach an analysis we used the crystal structure of tosylchymotrypsin as a starting point; hydrogens were placed on the heavy atoms whose positions are defined by the x-ray work and bad contacts between these removed by empirical conformational energy minimization using the standard force field of Polygen's CHARMM/QUANTA package (Version 19). A collection of the hydrogens closest to the center of the tosyl methyl group were used to define a model system and, assuming that this collection tumbles isotropically with a correlation time τ_c while the tosyl methyl group rotates diffusively with a correlation time τ_i, the results of spin-lattice relaxation time, linewidth, and various $^3H\{^1H\}$ NOE determinations for the species containing one, two, and three tritons in the tosyl methyl group were calculated. The program used is based on the Solomon equations (13) and was largely derived from one described previously (3). Standard formulations for the spectral density functions were used and rotation of protein methyl groups was included explicitly (4), but cross-correlation effects were neglected in these calculations (5). The computed behavior

for each tritiated species was summed appropriately, according to the fraction of each species present in the mixture and the amount of tritium present in each species, to produce the predicted composite behavior for the protonated/tritiated sample. In computing 2D NOE results chemical shifts corresponding to the random coil shifts of the protons of the enzyme were used (6). The linewidth for these protons was taken arbitrarily to be 38 Hz; this choice does not alter conclusions reached about relative peak intensities.

Tosylchymotrypsin is monomeric at pH 4.4 and is presumably monomeric under the conditions used in the present work (7). For monomeric enzyme, the rotational correlation time τ_c is approximately 15 ns (8). It is probable that the rotation of the methyl groups is rapid enough that the calculations are insensitive to the value of τ_i and we chose to use 0.02 ns (9).

Calculations were done with a model composed of the spins of the tosyl group plus the 39 non-exchangeable protons of the protein closest to the carbon of the tosyl methyl group and the spins of the three methyl groups closest to the tosyl methyl (methyls of Val-15, Met-188, and Val-109) since methyl groups act as relaxation sinks in proteins. Additional calculations were carried out with a model based on the 82 spins that are closest to the tosyl methyl carbon plus the 6 closest methyl groups. Comparison of these showed that the tritium linewidth, spin-lattice relaxation behavior, and steady state $^3H\{^1H\}$ NOE are almost completely defined by the geometry and dynamics of spins directly adjacent to the tosyl methyl group while the time-dependence of the 2D NOEs is dependent on the nature of the entire proton lattice. The relaxation behavior of spins at the edges of the model is not correctly described in the model since these spins are missing critical interactions with spins which are nearby in the protein but are not included because they are beyond a cutoff distance. To account for these missing interactions an additional (external) spin-lattice relaxation contribution (0.5 s) was added for each of the spins on the periphery of the model.

Calculations of T_1, lineshape, and the steady state $^3H\{^1H\}$ NOE using the procedures outlined above gave results in good agreement with experiment (Table I), considering the approximations made and the neglect of conformational motions of the protein structure. However, as comparison of Figures 1 and 2 demonstrates, the predicted 2D NOE behavior as a function of mixing time was not in agreement with experiment. The intensities of the 2.2 and 7.0 ppm features of the 2D cross sections act as intensity/distance standards to be compared to intensities of the remaining features in the cross sections that arise from interactions of the tritium nuclei with protons of the protein. The predicted intensities for these are too large and we found that no reasonable adjustment of the correlation times used in the calculation would improve these relative intensities while retaining the agreement between observed and calculated T_1, T_2, and steady state NOEs.

The disagreement between the experimental $^3H\{^1H\}$ 2D NOEs and those calculated based on the structure of tosylchymotrypsin in the solid state must arise because the interaction distances between the protons of the tosyl binding site and the nuclei of the tosyl methyl group are not correct in the model and the situation in solution must be such that the tosyl methyl group can move away from these contacts. A reasonable pathway for breaking these interactions would involve movement of the tosyl group out of the tosyl pocket. Extensive computer graphics and adiabatic mapping studies of this possibility showed that a series of rotations about the single bonds along the sidechain of Ser-195 and within the tosyl group would provide a low energy pathway for motion of the tosyl group away from the active site pocket and toward a more solvent-exposed position. A series of simulations was done for a structure with τ_c = 15 ns and τ_i = 0.02 ns in which the tosyl group was maximally extended away from the protein. In this conformation only intra-tosyl group interactions are apparent in the computed 2D NOE results and it is only at long mixing times that cross peak intensities for protein-tosyl interactions begin to appear through spin diffusion effects.

Table I. Observed and Calculated Tritium Relaxation Behavior[a]

	Exp.	Calc.[b] *100% in*	Calc.[b] *50% in*	Calc.[b] *100% out*
T_1, s	0.53	0.36	0.50	0.61
Linewidth, Hz	31	30	29	28
$^3H\{^1H\}$ NOE[c]	-0.79	-0.81	-0.80	-0.77

a Enzyme inactivated with a sample of II in which the proportion of the CH2T, CHT2, and CT3 species 50:46:4.
b Computed values obtained using the models and parameters described in the text. The composite linewidths include an additional 3 Hz broadening to account for apodization and instrumental linewidth effects.
c Equilibrium NOE produced by constant irradiation of protons at high power.

Based on the intensities of the cross peaks observed in the 2D NOE spectra, we suggest that the tosylchymotrypsin structure in which the tosyl group completely occupies the active site pocket in the manner found in the crystal structure ("tosyl in" structure) and the tosylenzyme structure in which the tosyl group has moved away from any contact with the enzyme ("tosyl out" structure) represent two extremes in a system that is conformationally mobile. A third series of computer simulations in which the "tosyl in" and "tosyl out" structures were assumed to both be present in equal amounts but in rapid dynamic equilibrium gave predicted T_1, T_2, and $^3H\{^1H\}$ NOE behaviors that were in good agreement with experiments, *including satisfactory agreement with 2D NOE crosspeak intensities.* There can, of course, be a larger

number of conformations in equilibrium and to be consistent with our experimental observations it is only required that the rate of interchange between conformations be rapid enough to average NOE-producing interactions and that the mix of conformations present permit the tosyl group to reside within the tosyl pocket about 50% of the time.

DISCUSSION

The results and conclusions of the present study are consonant with nmr observations made with deuterium and carbon-13 labeled forms of tosylchymotrypsin (10). Consideration of linewidths at pH 4 for the tosylenzyme containing a CD_3 or $^{13}CH_3$ group led to the conclusion that the correlation time τ_c for the protein is 10-15 ns, a result consistent with the analysis of tritium relaxation behavior discussed above. Consideration of the deuterium linewidths for enzyme specifically deuterated at the tosyl aromatic ring positions indicated that the correlation time for the aromatic ring is only about 0.5 ns at pH 4, indicating that there is considerable mobility of the aromatic ring of the tosyl group beyond that provided by tumbling of the protein. Rotation of the tosyl ring is surely slow when the ring occupies the tosyl pocket because of the close protein contacts involved and τ_c for the aromatic deuterons in this environment must be close to that for overall tumbling of the protein (15 ns). By moving of the tosyl group out of this pocket, as suggested by the tritium nmr results described above, much freer rotation of the aromatic ring would be possible. A reduced local correlation time for the ring would arise by mixing of the slow motion characteristic of the "tosyl in" conformation and those motions which are present in accessible "tosyl out" structures.

EXPERIMENTAL

Tritium gas was purchased from Oak Ridge National Laboratory and contained 97.9% T_2 with the major contaminant being 1.76% DT. Deuterium oxide (99.9% atom % D), chlorine gas, *p*-toluenesulfonyl fluoride, and 10% Pd on carbon catalyst were purchased from Aldrich Chemical Co. α-Chymotrypsin was obtained as the 3X recrystallized, lyophylized product from Sigma Chemical Co. and was used without further purification.

4-Dichloromethylbenzenesulfonyl fluoride was prepared by treating a solution of 4.0 g (230 mmol) of *p*-toluenesulfonyl fluoride and 0.04 g of benzoyl peroxide in 230 mL of CCl_4, contained in a 3-neck round bottom flask, with Cl_2 gas at room temperature. The reaction was irradiated with a commercial sun lamp (200 W) 15 cm from the reaction flask for 3 h. The reaction was monitored by analyzing aliquots with capillary gc. It was observed that shorter reaction times lead to an increased proportion of monochlorinated product while longer times lead to only trace amounts of trichlorinated products and predominant amounts of an insoluble side product which was not fully characterized. The desired product was purified by

silica gel chromatography for an isolated yield of 23%, mp 77-80°C; ^{1}H NMR ($CDCl_3$, 500MHz) δ 8.07,8.04, 7.84 and 7.82 (AA'BB', 4H, ring) δ 6.74 (s, 1H, $CHCl_2$); IR (KBr) υ_{max} (SO_2F) 1410 and 1211 cm^{-1}, C-Cl 787 cm^{-1}; MS (m/z, relative intensity) 207, (M+-H^{37}Cl, 100.0), 209 (M+-H^{35}Cl, 37.1).

Catalytic tritiations of 4-dichloromethylbenzenesulfonyl fluoride to prepare tritium labeled tosyl fluoride (**II**) were carried out inside a glove box using a custom-built micro-hydrogenation apparatus. The reaction vessel was charged with a solution of 7.75 mg (32 μmol) of 4-dichloromethylbenzenesulfonyl fluoride and 10 μL of triethylamine in 3 mL of CH_3CN. The catalyst spoon of the apparatus was loaded with 7.8 mg of 10% Pd on carbon and the entire apparatus exhaustively degassed by the application of several freeze-pump-thaw cycles. Tritium gas was admitted *via* stainless steel vacuum lines to a pressure of 550 mm Hg over the frozen solution and the solution was then allowed to thaw. The pressure in the vessel was adjusted to 760 mm Hg and the catalyst then added to the solution. The mixture was stirred at room temperature. The reaction was monitored by the uptake of T_2 gas and also by removing small aliquots of the reaction mixture with a syringe followed by analysis using radio-TLC on precoated silica gel plates using hexane/ether (5:1) as the eluent. After 1 h the reaction mixture was frozen with liquid nitrogen and the remaining tritium pumped away. The system was flushed with nitrogen gas and the solution thawed. The catalyst was removed by filtration and the solvent stripped by evaporation with a stream of dry nitrogen gas. (Gentle conditions were used for solvent removal because of the high vapor pressure of the product and the attendant potential for product loss by sublimation.) The residue was taken up in 1.7 mL of CD_3CN for analysis by NMR. Subsequent liquid scintillation counting showed that the product contained from 1.0 to 1.5 Ci. The scintillation results could be somewhat misleading since there could be some tritiated impurities left in the product from reduction of the solvent. The yield of product was not determined but was at least sufficient to give rapid and complete inactivation of the enzyme in the procedure described below.

General Procedures. Radio-thin layer chromatograms were analyzed using a Varian Aerograph Radio Scanner and a Hewlett Packard 3390A integrator. Gas chromatography was run on a Hewlett Packard 5890 with a 3392A integrator. An Ultra II column (5% phenylmethyl silane 25M x 0.200mm) was used isothermally at 150°C with He carrier gas. Infra-red spectra were obtained with a Bio-Rad/Diglab FTS-60 with the samples contained in KBr pellets. Carbon and proton NMR spectra were run on a General Electric GN500 with $CDCl_3$ as solvent and are referenced to tetramethylsilane.

Inhibition of Enzyme. One hundred twenty three mg (4.9 μmoles) of α-chymotrypsin was dissolved in 15 mL of 0.05 M phosphate buffer adjusted to pH 7. Tritiated inhibitor (product

of *ca.* 30 μmole reaction described above) was taken up in 1.5 mL CH_3CN and added to the enzyme solution. The resulting mixture was incubated at room temperature for several hours; loss of enzyme activity was monitored by hydrolysis of the substrate N-glutaryl-phenyl-alanine 4-nitroanilide (11). The inactivation reaction was halted when the activity had been reduced to less than 1% of its initial value.

Purification of tritiated tosylchymotrypsin. The solution of inactivated enzyme was loaded into an Amicon Centriprep-10 concentrator cell (10 kDal cutoff) for removal of excess inactivator and for solvent exchange with deuterated buffer. The solution was centrifuged through the filter to a volume of 3 mL, then diluted to 6 mL with 0.05 M KCl in D_2O and recentrifuged to 3 mL. This cycle was repeated 9 times with the final centrifugation going to a volume of 1.5 mL. The final sample had a calculated solvent deuterium level of greater than 99.8% and no free inhibitor detectable by NMR. Sample pH was adjusted to 4.0 ± 0.1 with microliter amounts of 1.0 M HCl in D_2O. The final protein concentration was 1.1 mM as determined spectrophotometrically at 282 nm using a molar extinction coefficient for the protein of $5x10^4$ M-1 cm-1 (12). The final sample radioactivities as determined by liquid scintillation counting were in the range of 60 to 80 mCi, with the average specific activity for the samples being 45 Ci/mmol.

Tritium NMR Spectroscopy. Enzyme samples (1.5 mL) were placed in Teflon tubes (Wilmad No. 6010) which were then placed inside a standard 10 mm glass NMR tube. Tritium NMR spectroscopy was carried out at 320 MHz on an IBM/Bruker AF-300 spectrometer. All spectra were obtained with the sample spinning and with the sample temperature regulated at 297°K by the instrument controller.

Spin-lattice relaxation times (T_1) were determined by the inversion recovery method with a composite 180° pulse and were fit to a three-parameter function using a routine in the spectrometer software. The estimated error for the T_1 values obtained is ±10%. Transverse relaxation times (T_2) were estimated by fitting the observed ^3H lineshapes of the inactivated enzyme with a composite of Lorentzian lines and are believed to be reliable to ±20%. Equilibrium (steady state) $^3H\{^1H\}$ nuclear Overhauser effects (NOEs) were determined by comparison of the integrations of the spectra obtained with and without proton irradiation during the pre-acquisition period. Two-dimensional $^3H\{^1H\}$ heteronuclear NOE spectra (14) were obtained using the TPPI method to provide quadrature detection in the t_1 dimension (15). Typically, 512 transients were collected for each of 64 t_1 values with the delay between collections being greater than 5 times T_1 such that, given the observations in transient NOE experiments, the tritium magnetization would be fully recovered before the start of the next scan. The total time for a 2D NOE experiment was about 60 hr. The 2D data sets spanned 3000 Hz

(10 ppm) in the ^1H dimension, represented by 64 points initially. In processing, the ^1H dimension was zero filled to 1024 points. The ^3H dimension in the NOESY spectra was referenced to the chemical shift of the methyl signal of the free inactivator at 2.44 ppm (protonated) or 2.43 ppm (deuterated). No adjustments were made for potential protein induced chemical shifts in the bound inactivator as these will likely be small.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant GM-25975. We are indebted to Professor T. C. Bruice for access to his Silicon Graphics computer system, to Dr. D. H. Gregory for his assistance with some of the calculations, to Mr. H. Morimoto for his invaluable assistance with the tritiation reactions, and to Dr. E. Olejniczak for providing a copy of his program.

REFERENCES

1. Birktoft, J. J.and Blow, D. M. - *J. Mol. Biol.* **68**: 187-240 (1972).
2. Bothner-By, A. A. - Biological Applications of Magnetic Resonance (Shulman, R. G., Ed), Academic, New York, 1979; p. 208.
3. Gerig, J. T., Luk, K. F. S. and Roe, D. C. - *J. Am. Chem. Soc.* **101**: 7698-7704 (1979).
4. Olejniczak, E. T. - *J. Magn. Reson.* **81**: 392-394 (1980)
5. Bull, T. E. - *J. Magn. Reson.* **72**: 397-413 (1987).
6. Bündi, A. and Wuthrich, K. - *Biopolymers* **18**: 285-297 (1987).
7. Horbett, T. A. and Teller, D. C. - *Biochemistry* **12**: 1349-1358 (1973).
8. (a) Kuznetsov, A. N., Ebert, B. and Gyul Khandanyan, G. V. - *Mol. Biol. (Moscow)* **9**: 871 (1975); (b) Vaz, W. L. C. and Schoellmann, G. - *Biochim. Biophys. Acta* **439**: 206-218 (1976).
9. Gerig, J. T., Loehr, D. T., Luk, K. F. S. and Roe, D. C. - *J. Am. Chem. Soc.* **101**: 7482-7487 (1979).
10. Ando, M. E., Gerig, J. T. and Weigand, E. F. - *J. Am. Chem. Soc.* **104**: 3172-3178 (1982).
11. Erlanger, B. F., Edel, F. and Cooper, A. G. - *Arch. Biochem. Biophys.* **115** 206-210 (1966).
12. Neurath, H. and Schwert, G. - *Chem. Rev.* **46**: 69-153 (1950).
13. Solomon, I. - *Phys. Rev.* **99**: 559-565 (1955).
14. Kövér, K. E. and Batta, G. - *Prog. NMR Spectrosc.* **19**: 223-266 (1987).
15. Bodenhausen, G.; Kogler, H.; Ernst, R. R. - *J. Magn. Reson.* **58**: 370-388 (1984).