

Elimination of spin diffusion effects in saturation transfer experiments: application to hydrogen exchange in proteins

Malene Ringkjøbing Jensen, Søren M. Kristensen and Jens J. Led*

Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

Received 6 September 2006; Revised 23 November 2006; Accepted 11 December 2006



The NMR saturation transfer experiment is widely used to characterize exchange processes in proteins that take place on the ms-s timescale. However, spin diffusion effects are inherently associated with the saturation transfer experiment and may overshadow the effect of the exchange processes of interest. As shown here, the effects from spin diffusion and exchange processes can be separated by varying the field strength of the saturation pulse, thereby allowing correct exchange rates to be obtained. The method is demonstrated using the hydrogen exchange process in the protein *Escherichia coli* thioredoxin as an example. Copyright © 2007 John Wiley & Sons, Ltd.

Supplementary electronic material for this paper is available in Wiley InterScience at <http://www.interscience.wiley.com/jpages/0749-1581/suppmat/>

KEYWORDS: NMR; ^1H ; ^{15}N ; saturation transfer; spin diffusion; hydrogen exchange; thioredoxin

INTRODUCTION

The saturation transfer experiment^{1,2} is widely used in nuclear magnetic resonance (NMR) studies of dynamical events in proteins. In particular, the saturation transfer experiment is useful for characterizing exchange processes, such as the exchange between different conformations of a protein, the exchange of the amide protons with the solvent water,^{1–10} or the exchange between a ligand-bound and a free protein.^{11,12}

The saturation transfer experiment requires that individual signals are observed for each of the exchanging species. The experiment is usually carried out by saturating an NMR signal of one of the exchanging species and monitoring the effect on the corresponding signal of the other species caused by the exchange-mediated transfer of saturated magnetization. If the rate of exchange is comparable to the longitudinal relaxation rates of the involved nuclei (normally on the ms-s timescale), the rate of exchange can be derived from the change in intensities of the monitored signals. However, in proteins the saturation of individual ^1H resonances is often hampered by spectral overlap, which gives rise to saturation of other adjacent signals. This unintentional saturation creates a problem since it spreads rapidly to the entire protein molecule by dipolar cross relaxation (spin diffusion) through the magnetically connected network of adjacent ^1H spins in the protein. Consequently, it becomes difficult to separate the effect of spin diffusion from the effect of exchange.

Here, we examine in detail the influence of spin diffusion in proteins on the results of the saturation transfer experiment. The exchange of the amide protons in *Escherichia coli* thioredoxin with the solvent water is used as a model system. The exchange process is monitored through the decrease of the amide proton intensities, which is observed after presaturating the solvent resonance for a few seconds. We show that the decrease in the amide proton intensities due to spin diffusion can be comparable to the intensity reduction caused by fast amide proton exchange on the ms-s timescale. Furthermore, we present a method that allows separation of the effect of spin diffusion from the effect of hydrogen exchange. The method relies on a variation of the field strength, B_1 , of the pulse used for saturating the solvent resonance, and exploits the fact that the effect of spin diffusion on the amide proton intensities is more dependent on the strength of B_1 than is the effect of the solvent exchange. Finally, we discuss limitations and the applications of the method.

EXPERIMENTAL

NMR samples

The protein *E. coli* thioredoxin (Trx)¹³ was used as a model protein since the hydrogen exchange in both reduced and oxidized Trx is well characterized by means of the deuterium exchange and the saturation transfer techniques.¹⁴ The structure of Trx has previously been solved both by NMR spectroscopy¹⁵ and X-ray crystallography,¹⁶ showing that Trx consists of a β -sheet core surrounded by four α -helices.¹⁵

Cloning, expression, and purification of the ^{15}N -labeled model protein were performed as described previously.¹⁷ A

*Correspondence to: Jens J. Led, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK – 2100 Copenhagen Ø, Denmark. E-mail: led@kiku.dk

1.0 mM sample of oxidized Trx in 50 mM NaCl and 10% D₂O/90% H₂O at pH 7.0 was sealed under nitrogen and used in the hydrogen exchange studies as described below.

NMR experiments

All NMR experiments were carried out on a Varian Unity Inova 500 spectrometer equipped with a cold probe. The saturation transfer experiments were carried out at two different temperatures, 298 and 308 K, by applying a 2.5 s saturation pulse to the water resonance at $\delta_{\text{sat}} = 4.774$ ppm (298 K) or $\delta_{\text{sat}} = 4.654$ ppm (308 K). Subsequently, the amide proton intensities were detected in a ¹H-¹⁵N HSQC spectrum.¹⁸ A reference spectrum was recorded with the saturating field applied at $\delta_{\text{sat}} = -3.9$ ppm, where no protein signals resonate. The field strength, B_1 , of the saturation pulse was varied between 7 Hz and 140 Hz. The saturation transfer experiments were collected with 1024 complex t_2 data points and 150 complex t_1 data points, and a sweep width of 10 kHz in the ¹H dimension and 2 kHz in the ¹⁵N dimension. The CLEANEX-PM experiment¹⁰ was recorded with 1024 complex t_2 data points and 150 complex t_1 data points, and a sweep width of 10 kHz in the ¹H dimension and 2 kHz in the ¹⁵N dimension. The CLEANEX-PM experiment was carried out at 298 K. All signal intensities were obtained by a least-squares fitting procedure as described previously¹⁹ using the program Fuda.²⁰

RESULTS AND DISCUSSION

Spin diffusion effects in saturation transfer experiments

Spin diffusion effects are inherently associated with protein saturation transfer experiments.^{7,21} In addition to the decrease in intensity caused by hydrogen exchange, the saturation of the solvent water signal and the proximate α -proton resonances may also significantly attenuate the amide proton intensities through spin diffusion.⁷ Further attenuation of the amide proton intensities can occur through nuclear Overhauser enhancements (NOEs) caused by direct dipole-dipole interactions between the amide protons of the protein and water molecules temporarily associated with the protein,^{22,23} or via spin diffusion from nearby labile hydroxyl protons that are saturated due to fast exchange with the solvent.^{7,24}

Figure 1 shows the results of a saturation transfer experiment carried out on the model protein Trx at 298 K. The figure shows a per residue comparison of the amide proton intensity attenuation factor, Γ , where Γ is defined as

$$\Gamma = \frac{M^{\text{sat}}}{M^{\infty}} \quad (1)$$

Here, M^{sat} is the intensity of the amide proton signals in the HSQC spectrum recorded with solvent saturation ($\delta_{\text{sat}} = 4.774$ ppm), while M^{∞} is the corresponding intensity in the reference spectrum ($\delta_{\text{sat}} = -3.9$ ppm). Thus, $\Gamma = 1$ for amide protons in slow exchange with the solvent, while $\Gamma < 1$ for amide protons exchanging on the ms-s timescale. As shown in Fig. 1, Γ is significantly smaller than 1 for all residues in Trx. However, since, for instance, the ¹H-¹⁵N

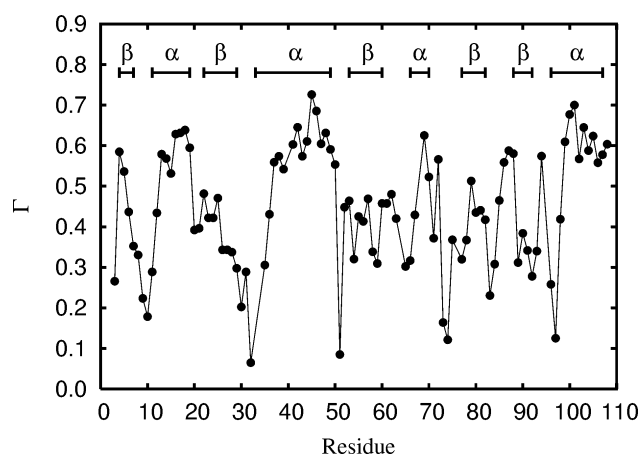


Figure 1. Variation of the amide proton intensity attenuation factor, Γ , with the residue number in Trx. The uncertainties of the attenuation factors are comparable to or smaller than the size of the symbols. The saturation frequency was $\delta_{\text{sat}} = 4.774$ ppm, and the strength of the saturating field was $B_1 = 140$ Hz.

cross peaks of the residues, I23, L24, V25, L79, L80, and F81, are still present in the HSQC spectrum 723 h after transfer of the protein into D₂O,¹⁴ amide proton exchange on the ms-s timescale cannot be the cause of all $\Gamma < 1$ observed in Fig. 1. This suggests that the amide proton intensities are affected by spin diffusion associated with the saturation of the solvent resonance.

To investigate the extent of spin diffusion in Trx as compared to the effect of hydrogen exchange, a series of saturation transfer experiments were carried out at different saturation frequencies, δ_{sat} . Figure 2 shows the results of two of these experiments with (A) $\delta_{\text{sat}} = 2.1$ ppm and (B) $\delta_{\text{sat}} = 3.1$ ppm. The saturation at these frequencies leaves the solvent resonance practically unperturbed and the effects from hydrogen exchange can, therefore, be neglected. Yet, the amide proton intensities are greatly reduced due to spin diffusion throughout the protein, as shown in Fig. 2. Furthermore, the change in the saturation frequency, δ_{sat} , from 2.1 ppm to 3.1 ppm, results in rather different attenuation patterns, which shows that the intensity reduction caused by spin diffusion depends strongly on the chemical shifts of the protons, and on the three-dimensional structure of the protein. Moreover, a comparison of Figs 1 and 2 shows that the reduction of the amide proton signals caused by spin diffusion may be comparable to the reduction caused by amide proton exchange on the ms-s timescale.

Separating hydrogen exchange from spin diffusion effects

Because of the spin diffusion effects, the amide protons that are affected by exchange on the ms-s timescale cannot be identified unambiguously from the data presented in Fig. 1. However, exchange and spin diffusion effects may be distinguished by performing saturation transfer experiments at two temperatures. Thus, exchange effects increase with increasing temperature, while spin diffusion effects in the slow-tumbling regime decrease with increasing temperature. Hence, the ratio of the attenuation factors, $\Gamma^{T_a}/\Gamma^{T_b}$, obtained

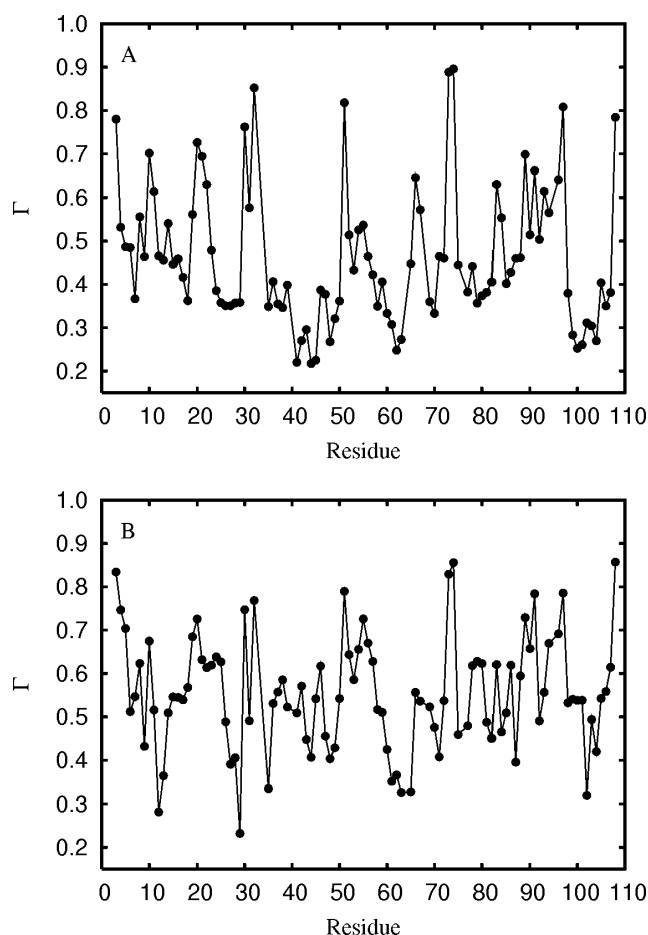


Figure 2. Variation of the amide proton intensity attenuation factor, Γ , with the residue number in Trx. The uncertainties of the attenuation factors are comparable to or smaller than the size of the symbols. The strength of the saturating field was $B_1 = 140$ Hz. The saturation frequency was $\delta_{\text{sat}} = 2.1$ ppm (A) or $\delta_{\text{sat}} = 3.1$ ppm (B).

at two temperatures $T_a > T_b$, will be smaller than 1 when the exchange process dominates, and larger than 1 when spin diffusion effects dominate. Figure 3 shows the ratio, $\Gamma^{308\text{ K}}/\Gamma^{298\text{ K}}$, for Trx as a function of the residue number. The data correlate well with the results of the CLEANEX-PM experiment¹⁰ carried out at 298 K. The CLEANEX-PM experiment provides a ^1H - ^{15}N HSQC spectrum with peaks arising solely from amide groups with ^1H exchange on the ms-s timescale, while effectively suppressing the spurious peaks arising from artifacts such as intramolecular NOEs and magnetization transfer from α -protons coincident with the water resonance. On the basis of the CLEANEX-PM experiment, and in agreement with previous deuterium exchange data,¹⁴ 28 amide protons in Trx were found to be in fast exchange with the solvent (filled circles Fig. 3). The majority of the amide protons, which are in fast exchange with the solvent according to the CLEANEX-PM experiment, show $\Gamma^{308\text{ K}}/\Gamma^{298\text{ K}}$ ratios below 1.

Even if the temperature dependence of the intensity attenuation factor, Γ , of the amide protons may reveal whether spin diffusion or hydrogen exchange dominates, Γ still depends on both effects, and the spin diffusion may still mask the presence of exchange. However, as shown below,

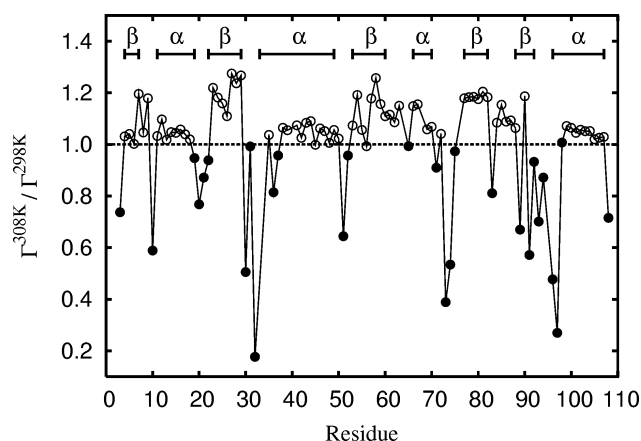


Figure 3. The ratio of the amide proton intensity attenuation factors, Γ , at 298 K and 308 K, as a function of the residue number in Trx. The uncertainties of the ratios are comparable to the size of the symbols. The saturation frequency, δ_{sat} , was 4.774 ppm and 4.654 ppm at 298 K and 308 K, respectively. The strength of the saturating field was $B_1 = 140$ Hz. Amide protons, which exchange on the ms-s timescale according to the CLEANEX-PM experiment,¹⁰ are indicated by filled circles.

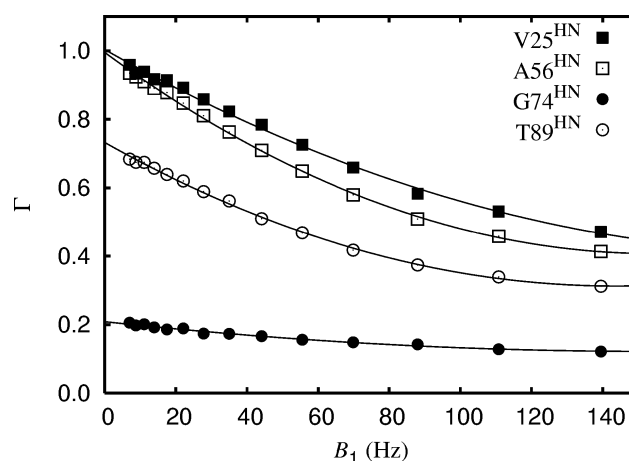


Figure 4. The amide proton intensity attenuation factor, Γ , for four different amide protons, as a function of the strength of the saturating field, B_1 . The uncertainties of the attenuation factors are comparable to the size of the symbols. The saturation frequency was $\delta_{\text{sat}} = 4.774$ ppm.

the two effects respond differently to a change in the strength of the saturating field. Figure 4 shows the dependence of Γ on the strength, B_1 , of the saturating field for four different amide protons, V25^{HN} , A56^{HN} , G74^{HN} , and T89^{HN} . As B_1 approaches zero, spin diffusion becomes less pronounced because the saturating pulse becomes more selective. Thus, for amide protons affected only by spin diffusion, Γ approaches 1 as B_1 goes to zero. On the other hand, if the amide protons are affected by hydrogen exchange on the ms-s timescale, Γ approaches a value smaller than 1 as B_1 goes to zero. Figure 4 shows that V25^{HN} and A56^{HN} are affected by the saturating pulse only through spin diffusion, while G74^{HN} and T89^{HN} exhibit hydrogen exchange on the ms-s timescale.

A corrected amide proton intensity attenuation factor, Γ^{cor} , unaffected by spin diffusion can be obtained by extrapolating the data in Fig. 4 to $B_1 = 0$. Since each

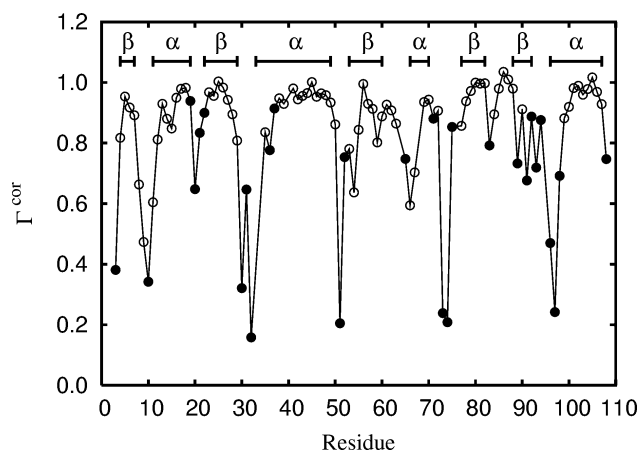


Figure 5. The amide proton intensity attenuation factor, Γ , corrected for spin diffusion as a function of the residue number in Trx at 298 K. The uncertainties of the attenuation factors are comparable to or smaller than the size of the symbols. Amide protons, which exchange on the ms-s timescale according to the CLEANEX-PM experiment,¹⁰ are indicated by filled circles.

amide proton may be affected by several partially saturated α -protons through spin diffusion, the functional form of the curves in Fig. 4 is not easily derived. Therefore, the extrapolation was done assuming that the data follow a second order polynomial

$$\Gamma(B_1) = aB_1^2 + bB_1 + \Gamma^{\text{cor}} \quad (2)$$

where a , b , and Γ^{cor} are constants that are obtained by a least-squares fit to the measured intensities of each amide proton as a function of the B_1 field strength. The solid curves in Fig. 4 correspond to the least-squares fits.

Figure 5 shows the corrected amide proton intensity attenuation factor, Γ^{cor} , as a function of the residue number in Trx. It is seen that many of the amide protons now show an attenuation factor close to 1, indicating that no exchange on the ms-s timescale takes place. Furthermore, $\Gamma^{\text{cor}} < 1$ for amide protons located in loop regions or in the beginning of α -helical structures where the hydrogen exchange is expected to be fast.

Limitations of the method

If the resonance frequency of a proton, such as an α -proton, coincides with the water resonance and hence the frequency of the saturation pulse, the proton will be saturated even at the lowest field strength applied here ($B_1 = 7$ Hz). Therefore, the extrapolation described above fails for the amide protons spatially close to protons that resonate at the water chemical shift. For example, as seen in Fig. 5, the attenuation factors of L53^{HN} and T54^{HN} are significantly smaller than 1 even though the hydrogen exchange rates of these amide protons are of the order 10^{-5} s^{-1} , as shown previously.¹⁴ The α -proton of residue L53 has a chemical shift of 4.77 ppm at pH 7.0 and 298 K, which is identical to the chemical shift of water, while T54^{HN} has a strong sequential NOE to the α -proton of L53.

This problem can be alleviated by recording the spectra at two different temperatures and taking advantage of

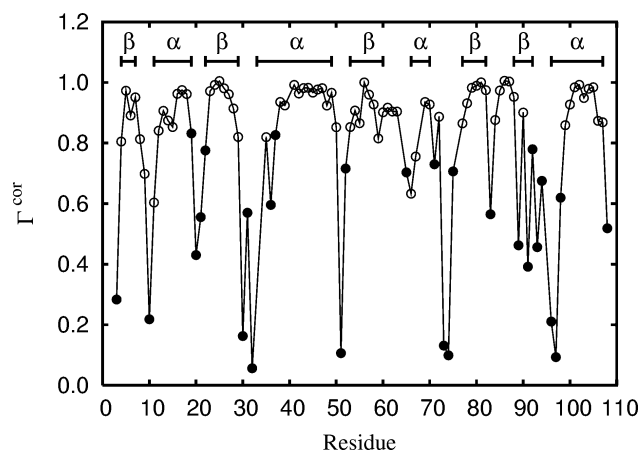


Figure 6. The amide proton intensity attenuation factor, Γ , corrected for spin diffusion, as a function of the residue number in Trx at 308 K. The uncertainties of the attenuation factors are comparable to or smaller than the size of the symbols. Amide protons, which exchange on the ms-s timescale according to the CLEANEX-PM experiment,¹⁰ are indicated by filled circles.

the strong temperature dependence of the water resonance. Figure 6 shows the results of the saturation transfer experiment at 308 K. By comparison with Fig. 5, it is seen that Γ^{cor} decreases for the amide protons in fast exchange with the solvent as the temperature is increased, in accordance with the increased exchange rates at higher temperatures. More importantly, the amide protons of L53 and T54 now appear as slowly exchanging at 308 K because the saturating field is applied at a chemical shift value different from that of the L53 α -proton.

As shown in Figs 5 and 6, the amide signals of the residues T8, D9, S11, T66, and A67 are also saturated even though the amide proton exchange process is slow.¹⁴ In this case, the amide proton signals are attenuated due to an NOE to the fast exchanging hydroxyl protons as noted previously for serine and threonine,⁷ and tyrosine residues.²⁴ In general, an unwanted saturation of amide protons spatially close to fast exchanging protons cannot be prevented by a change in temperature.

Applications

Previously, it has been shown that amide hydrogen exchange effects can be separated from spin diffusion (cross relaxation) effects using saturation transfer experiments at different pH values.⁷ In contrast, the approach presented here can be applied at a single pH value. This is particularly valuable in the study of proteins that are stable only in a narrow pH range, or in the case of proteins that change conformation with pH. Furthermore, this method does not assume a specific model for the exchange process, such as a simple two-site exchange model, but can be applied also to exchange processes that take place according to more complicated exchange models. In this context it should be noted that the exchange process of fast exchanging amide protons in proteins has been suggested to take place according to more sophisticated models, such as the Linderstrøm-Lang model.^{9,25,26}

The approach presented here can be applied also to other kinds of exchange processes in proteins than amide hydrogen exchanges. An example is the exchange between two distinct conformations of a protein, where each conformation gives rise to separate signals in the NMR spectrum, as for instance the exchange between a folded and an unfolded protein,²⁷ or the exchange between a metal-free and a metal-bound protein.^{17,28} Also, in combination with the CLEANEX-PM experiment, the method presented here may be used to identify amide protons close to fast exchanging hydroxyl groups, and may provide distance information that is not available otherwise.

CONCLUSION

Our study shows that the applicability of the saturation transfer experiment for characterizing exchange processes in proteins is complicated by spin diffusion effects. However, the spin diffusion effects can be separated from the exchange contributions by measuring the effect of the saturation as a function of the field strength of the saturation pulse, and by performing experiments at two temperatures.

Supplementary material

Supplementary electronic material for this paper is available in Wiley InterScience at: <http://www.interscience.wiley.com/jpages/0749-1581/suppmat/>

Acknowledgements

We thank Mathias A. S. Hass for helpful discussions and Conni Lauritzen, Gitte Petersen and John Pedersen, Unizyme A/S, for providing the thioredoxin samples. This work was supported by the Danish Natural Science Research Council; (Contract/grant numbers: 9400351, 9801801, 51-00211, 21-01-0545, and 21-04-0519), Carlsbergfondet; (Contract/grant numbers: 1624/40). Novo Nordisk Fonden; (Contract/grant numbers: 2003-11-28) and Villum Kann Rasmussen Fonden; (Contract/grant numbers: 8.12.2003)

SUPPORTING INFORMATION

Tables listing the amide proton intensity attenuation factors at 298 K and 308 K in thioredoxin as a function of

the field strength of the saturation pulse are available at <http://www.spectroscopyNOW.com/nmr/supplementary>.

REFERENCES

1. Forsen S, Hoffman RA. *J. Chem. Phys.* 1963; **39**: 2892.
2. Krishna NR, Huang DH, Glickson JD, Rowan R, Walter R. *Biophys. J.* 1979; **26**: 345.
3. Waelder S, Lee L, Redfield AG. *J. Am. Chem. Soc.* 1975; **97**: 2927.
4. Led JJ, Gesmar H. *J. Magn. Reson.* 1982; **49**: 444.
5. Hvidt A, Gesmar H, Led JJ. *Acta Chem. Scand.* 1983; **B 37**: 227.
6. Led JJ, Gesmar H, Abildgaard F. *Methods Enzymol.* 1989; **176**: 311.
7. Spera S, Ikura M, Bax A. *J. Biomol. NMR* 1991; **1**: 155.
8. Gemmecker G, Jahnke W, Kessler H. *J. Am. Chem. Soc.* 1993; **115**: 11620.
9. Zheng Z, Gryk MR, Finucane MD, Jardetzky O. *J. Magn. Reson., Ser. B* 1995; **108**: 220.
10. Hwang TL, van Zijl PCM, Mori S. *J. Biomol. NMR* 1998; **11**: 221.
11. Mayer M, Meyer B. *Angew. Chem., Int. Ed. Engl.* 1999; **38**: 1784.
12. Nakanishi T, Miyazawa M, Sakakura M, Terasawa H, Takahashi H, Shimada I. *J. Mol. Biol.* 2002; **318**: 245.
13. Holmgren A. *Ann. Rev. Biochem.* 1985; **54**: 237.
14. Jeng MF, Dyson HJ. *Biochemistry* 1995; **34**: 611.
15. Jeng MF, Campbell AP, Begley T, Holmgren A, Case DA, Wright PE, Dyson HJ. *Structure* 1994; **2**: 853.
16. Katti SK, LeMaster DM, Eklund H. *J. Mol. Biol.* 1990; **212**: 167.
17. Jensen MR, Petersen G, Lauritzen C, Pedersen J, Led JJ. *Biochemistry* 2005; **44**: 11014.
18. Kay LE, Keifer P, Saarinen T. *J. Am. Chem. Soc.* 1992; **114**: 10663.
19. Kristensen SM, Sørensen MD, Gesmar H, Led JJ. *J. Magn. Reson., Ser. B* 1996; **112**: 193.
20. Kristensen SM. *Fuda: A Function and Data Fitting and Analysis Package*. Department of Chemistry, University of Copenhagen: Copenhagen, smk@kiku.dk, 2005.
21. Moy FJ, Scheraga HA, Patt SL, Montelione GT. *J. Magn. Reson.* 1992; **98**: 451.
22. Otting G, Wüthrich K. *J. Am. Chem. Soc.* 1989; **111**: 1871.
23. Otting G, Liepinsh E, Wüthrich K. *Science* 1991; **254**: 974.
24. Pitner TP, Glickson JD, Dadok J, Marshall GR. *Nature* 1974; **250**: 582.
25. Gryk MR, Finucane MD, Zheng Z, Jardetzky O. *J. Mol. Biol.* 1995; **246**: 618.
26. Jardetzky O, Finucane MD. *Mol. Phys.* 1998; **95**: 1127.
27. Farrow NA, Zhang O, Forman-Kay JD, Kay LE. *Biochemistry* 1995; **34**: 868.
28. Jensen MR, Led JJ. *Biochemistry* 2006; **45**: 8782.