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PASE (*PA*ramagnetic *S*ignals *E*nhancement): A New Method for NMR Study of Paramagnetic Proteins

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A new method for NMR spectra acquisition of paramagnetic proteins is described, based on the simple use of homonuclear broadband decoupling of the diamagnetic region. Several advantages are associated with this method which was applied to one-dimensional spectra, to 1D NOE-difference spectroscopy, and to 2D NOESY. The main advantage is a very flat baseline obtained using the PASE (*pa*ramagnetic *s*ignals *e*nhancement) method. Furthermore, the bulky region of the diamagnetic protons being suppressed, clean NOE-difference spectra can be acquired as well as improved 2D NOESY maps. Applications on 1D ^{1}H spectrum of bovine liver catalase (MW 230,000), and 1D and 2D on the high-spin form of the myoglobin, used as a model protein, are presented.

Key Words: **paramagnetic proteins; ^{1}H NMR; catalase; myoglobin.**

The study of paramagnetic biomolecules can be considered as a rather special field in NMR spectroscopy (*1–3*). Compared with diamagnetic proteins, specific conditions used for spectra acquisition as well as the objectives are often different. In some cases the main goal is to identify the nature of the metal ligands in addition to determining the electronic structure of the complex. This implies studying the hyperfine shifted signals in the coordination sphere close to the metal ion. The second goal is to determine the solution structure by assessing protons under moderate paramagnetic influence in the further coordination sphere. Most of these protons as well as the residues far from the metal can be assigned using standard techniques applied in the case of diamagnetic proteins. However, new tools are necessary in order to connect the fast relaxing signals with those of unperturbated protons. One method of assignment uses EXSY to correlate signals in both the paramagnetic and the diamagnetic forms. In addition to assignment, the EXSY experiment also allows the study of ligand exchange or electron transfer (*4*). However, for solution structure determination, the number of constraints based on NOE experiments is often low and two different strategies are used to increase the accuracy of the structures. One is based on the analysis of the proton longitudinal relaxation times (*5*) and the other uses the quantification of the isotropic shifts of strongly perturbated resonances (*6*). Nevertheless, an improvement of the quality of spectra would provide more information distinguishing between the protons close to the metal and those which are under weak paramagnetic influence.

Improvement in 2D acquisition has mainly been related to the adjustment of relaxation delays (and potentially mixing time) and of acquisition times $t_{2\max}$ and $t_{1\max}$. Recently, the use of WEFT-NOESY (*7*) was reported for the study of horseradish peroxidase in the low-spin state (*8*). At the same time, a new method called NOE-NOESY was published (*9*), based on the NOESY sequence with selective (on–off) saturation of a strongly shifted signal. The resulting spectrum, corresponding to a pseudo 3D experiment, was used for the study of myoglobin in the low-spin state.

We present the suppressing of the entire crowded diamagnetic region at the beginning of the sequence (*10*). Because the use of gradient is often very efficient in signal suppression, the first attempt was to use a 90° pulse followed by a pulse field gradient (PFG). In the case of high-spin hemoprotein, the hyperfine shifted signals are often characterized by very short T1 and T2. Consequently, the slow relaxing protons are suppressed whereas the fast relaxing signals can be recovered. In Fig. 1, different spectra of the met-aquo myoglobin are presented without baseline correction. The classical baseline distorsion associated with a large window of chemical shift was observed in the standard presaturation spectrum (Fig. 1a). Figure 1b shows the spectrum obtained after a PFG, used in order to purge the diamagnetic region. A drastic decrease of the 0–10 ppm region was observed, but a large remaining distorsion of the baseline was still present. The use of the super WEFT sequence (*11*) gave a better result in terms of baseline, but at the cost of a very disordered diamagnetic region (Fig. 1c). The spectrum in Fig. 1d corresponds to a simple homonuclear broadband decoupling using a WALTZ16 of 100 ms after a short presaturation irradiation of the residual water. Clearly,

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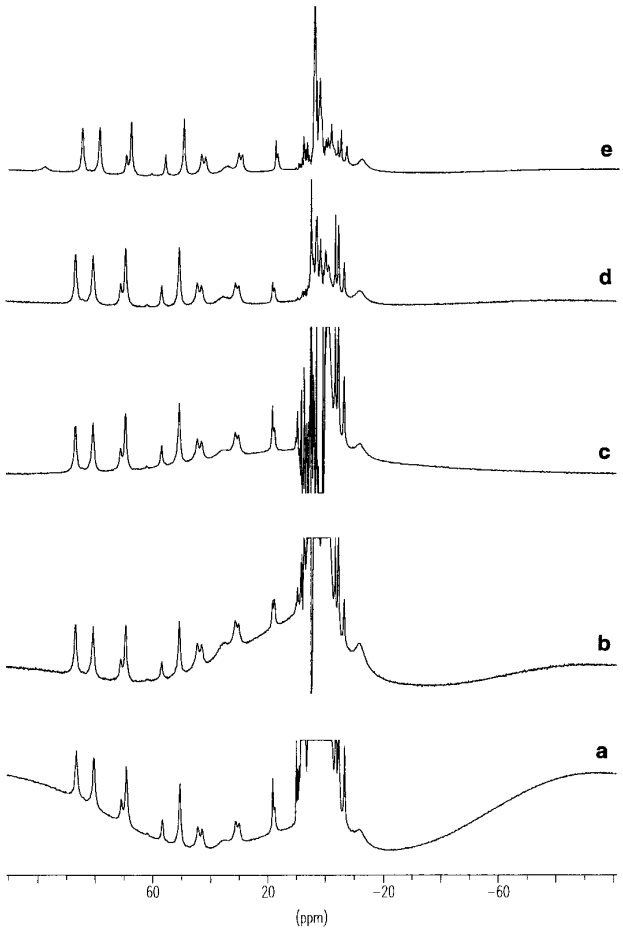

FIG. 1. Full 1D proton NMR spectra of sperm whale met myoglobin, 4 m*M* in deuterated phosphate buffer (except 1E), at 313 K. The spectra were acquired on a Bruker DMX 500 spectrometer, using 128 transients and identical acquisition time (19 ms) and repetition time (170 ms except in 1D 80 ms) but with different pulse sequences. (a) Presaturation of residual water; (b) suppression of the diamagnetic signal with a PFG, see text; (c) super weft sequence; (d) PASE method (50 ms presaturation, 100 ms decoupling, see text); (e) same as (d) but in 90% H_2O (the decoupler offset was set 1 KHz downfield from the water to achieve better water suppression). In all cases no baseline correction was applied.

without any correction, the *pa*ramagnetic *s*ignal *e*nhancement (PASE) method gave a spectrum with a very flat baseline, even in the case of a sample in 90% H_2O (Fig. 1e). A large increase for the receiver gain values was also observed but without any increase of the *S*/*N* ratio as previously described with the use of a selective-excitation pulse sequence (*12*).

Although the routines for automatic baseline correction have been greatly improved, good results often require manual definition of points between resolved resonances. An example of such difficulty is presented in Fig. 2a which corresponds to the spectrum of catalase, acquired with a simple presaturation of the residual water. A similar spectrum has previously been reported (*13*). Catalase is a 230,000 Da tetrameric hemoprotein in the high-spin form in the resting state. Obviously, baseline correction of the spectrum shown in Fig. 2a is not simple. However, the PASE method, giving the spectrum shown in Fig. 2b, presents several broad resonances corresponding to heme substituents in the 40–80 ppm region, as previously reported (*13*). However, a new broad signal was clearly seen at 115 ppm and was assigned to the meta protons of the proximate sixth ligand, the tyrosinate 256. This assignment is based on previously reported work on model compounds (*14*) and mutated myoglobins (*15*). The temperature dependence of this signal is shown in Fig. 2c. This resonance obeys Curie's law with an extrapolated intercept in the diamagnetic region.

As previously pointed out, NMR studies of paramagnetic proteins can give information about the nature of the metallic ion or spin state of the complex through the assignment of hyperfine shifted signals. In such a case the bulky region 0–10 ppm does not present any great interest. Thus, the suppression of these proton resonances associated with a significant enhancement of the quality of the baseline enables NMR studies of paramagnetic proteins of larger molecular weight.

A second application of the PASE method concerns the NOE-difference spectroscopy. As for every difference spectroscopy, careful setup is required. However, nice improvements over the last years have been obtained using a combination of selective pulses and PFG (*16*). Unfortunately, this sequence cannot be employed in the case of paramagnetic protons because of their short relaxation times. Myoglobin in its high-spin state was used

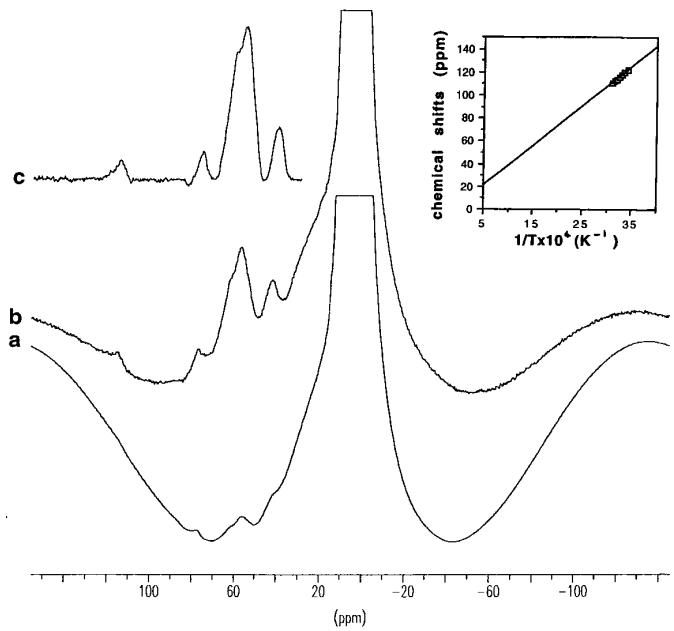

FIG. 2. Full 1D proton NMR spectra of bovine liver catalase, 0.75 m*M* in heme in deuterated borate buffer, at 323 K. The spectra were acquired, using 2K transients and identical acquisition time (7 ms) and repetition time (82 ms). (a) Presaturation of residual water; (b) PASE method (25 ms presaturation, 50 ms decoupling); (c) same as (b) but baseline corrected. The inset represents the chemical-shift dependence of the meta proton of the tyrosinate fifth ligand versus the inverse of the temperature.

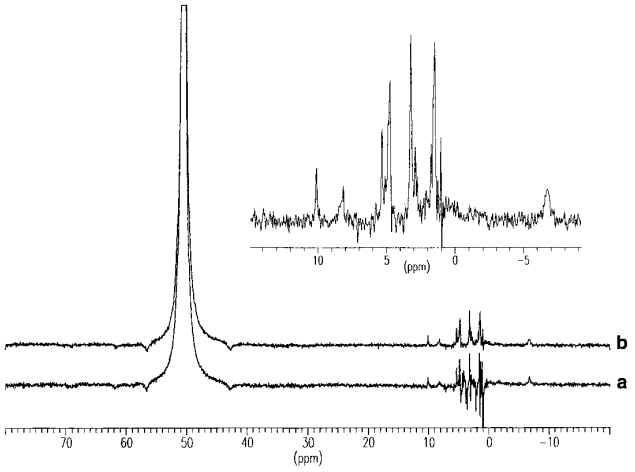

FIG. 3. NOE Difference spectrum of sperm whale met myoglobin, 4 m*M* in deuterated phosphate buffer, at 313 K. The spectra were acquired using 10K transients and an irradiation time of 25 ms. (a) Normal NOE diff. sequence with a presaturation of residual water of 225 ms; (b) PASE NOE diff. sequence with 25 ms of residual water and 200 ms of decoupling prior to the selective irradiation of 1-Methyl.

as an example to illustrate the improvement associated with the PASE method. Figure 3 shows the NOE spectra using a standard water-presaturated sequence (Fig. 3a) and the PASE method (Fig. 3b). The broadband decoupling applied at the beginning of each transient of the NOE-difference sequence is able to remove most of the protons under weak paramagnetic influence (Fig. 1d). Consequently, the difference between the on- and off- resonance data seems to be more accurate in light of the better quality of the spectrum in Fig. 3b in the 0–10 ppm region. It can be pointed out that the observed distorsions in the spectrum obtained with the standard water-presaturated sequence are related to the optimized conditions of acquisition for high-spin species (short irradiation time, high power level, and especially very short acquisition time). Such effects are routinely not observed in the case of conditions of acquisition adjusted for low-spin species. In spite of much progress in two-dimensional spectroscopy applied on paramagnetic proteins, the interest of 1D NOE-difference spectroscopy is well documented, especially in the case of high-spin complexes (*2*). The main advantage corresponds to the capacity to connect protons characterized by very large relaxation rates with neighboring protons relaxing slowly. This is possible because the length of irradiation time is free whereas the efficiency of the mixing time in a NOESY is under control of the relaxation rate. Because most of the protons under weaker paramagnetic influence resonate in the 0–10 ppm region we have found the PASE method to be a useful tool to detect such connectivities.

Of the artifacts limiting sensitivity in many types of two-dimensional NMR experiments, T1 ridges, T1 noise, and baseline distorsion have often been pointed out (*17*). A 2D PASE-NOESY of met-aquo myoglobin with a 5-ms mixing time and the diamagnetic suppression by broadband decoupling is visualized in Fig. 4. Obviously, the NOESY map is not symmetrical, the connectivities being observed only in the F2 dimension. Although the F1 dimension is often used in two-dimensional experiments because the cross peaks are less noisy than their counterpart in the F2 dimension, the resolution is better in this latter. This aspect is particularly relevant with spectrometers with digital acquisition which allows a smaller acquisition window, increasing F2 resolution. The spectrum in Fig. 4 presents clear connectivities between the hyperfine shifted signals and the protons in the diamagnetic region. Met myoglobin has been widely studied and all the strongly shifted resonances have been assigned based on isotopic labeling and NOEs (*18*). Preliminary analysis of the spectrum shown in Fig. 4 allows us to confirm all the previously assigned resonances with the exception of the two α-vinyl protons which should be reversed. Also, clear cross-peaks in the 0–10 ppm region allow us to complete the assignment of all heme proton resonances except the 6-β' propionate proton. At 313K, the two β-vinyl *trans* protons are resolved (*18a*), and strong cross-peaks are observed between the two sets of geminate CH_2 vinyl protons at $-6.99/2.08$ ppm and $-6.87/-0.52$ ppm. NOEs originating from the propionate protons are also labeled on Fig. 4; the chemical shifts of the heme protons are summarized in Table 1.

In conclusion we present a new tool for the study of paramagnetic metalloproteins, based on simple decoupling of the diamagnetic region by broadband decoupling or WALTZ16 sequence.

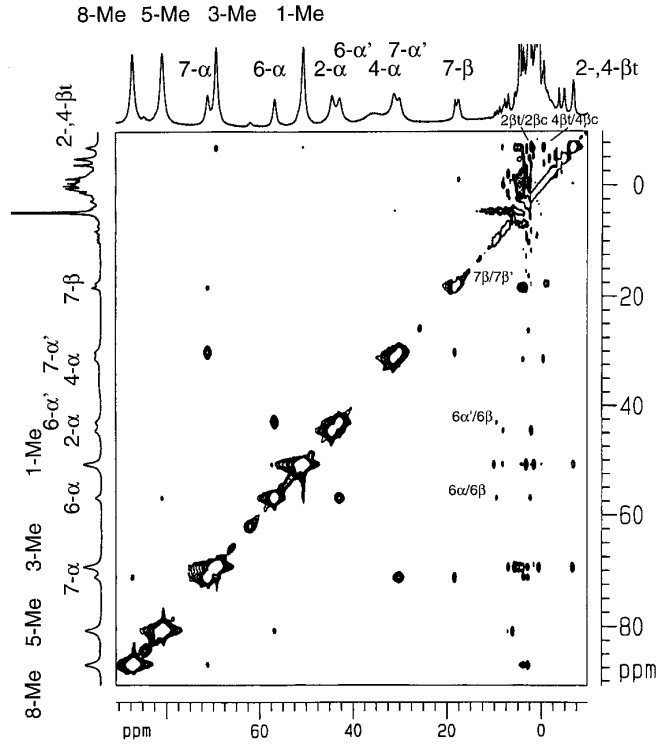

FIG. 4. PASE-NOESY spectrum of sperm whale met myoglobin, 4 m*M* in deuterated phosphate buffer, at 313 K. The spectrum was acquired in the TPPI mode using short presaturation of residual water (25 ms) followed by a broadband decoupling of 50 ms. A mixing time of 5 ms was used; 1600 transients were acquired for each of the 400 experiments.

TABLE 1
Heme Assignment of Sperm Whale Met Myoglobin in D_2O at 313 K

Heme substituents	Chemical shift (ppm)	Ref.
8-Me	87.15	*17*
5-Me	80.95	*17*
3-Me	69.38	*17*
1-Me	50.78	*17*
6-α	56.88	*17*
6′-α	43.03	*17*
6-β	9.58	
6′-β	n.d.	
7-α	71.24	*17*
7′-α	30.22	*17*
7-β-18.34	*17*	
7′-β	3.68	
2-vinyl α	44.58	
2-vinyl β *cis*	2.08	
2-vinyl β *trans*	−6.99	*17*
4-vinyl α	31.36	
4-vinyl β *cis*	−0.52	
4-vinyl β *trans*	−6.87	*17*

Note. n.d., not determined.

The main consequence is a real improvement of the baseline which gives a better visualization of severely broad-shifted resonances of large proteins. Enhancement of the quality of the NOE difference spectroscopy as well as better 2D NOESY maps is demonstrated. We believe this method is a useful tool for connecting the strongly hyperfine shifted signals with resonances occurring under the diamagnetic region. In the near future such an approach should be efficient in recording 3D spectra in order to facilitate assignment of resonances in dipolar interactions with protons under severe paramagnetic influence.

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REFERENCES

1. K. Wüthrich, *Structure Bonding* **8,** 53–121 (1970).
2. "Nuclear Magnetic Resonance of Paramagnetic Macromolecules" (G. N. La Mar, Ed.), Kluwer Academic, Dordrecht (1995).
3. I. Bertini, P. Turano, and A. J. Vila, *Chem. Rev.* **93,** 2833–2932 (1993).
4. (a)R. K. Gupta and A. G. Redfield, *Biochem. Biophys. Res. Commun.* **41,** 273–281, 1970; (b) R. K. Gupta and A. G. Redfield, *Science* **169,** 1204–1206 (1970).
5. (a)T. C. Pochapsky, X. M. Ye, G. Ratnaswamy, and T. A. Lyons, *Biochemistry* **33,** 6424–6432 (1994); (b) I. Bertini, I. C. Felli, C. Luchinat, and A. Rosato, *Proteins* **24,** 158–164, (1996); (c) J. G. Huber, J.-M. Moulis, and J. Gaillard, *Biochemistry* **35,** 12,705–12,711 (1996).
6. (a)S. D. Emerson and G. N. La Mar, *Biochemistry* **29,** 1556–1566 (1990); (b) M. Gochin and H. Roder, *Prot. Sci.* **4,** 296–305 (1995); (c) L. Banci, I. Bertini, G. G. Savellini, A. Romagnoli, P. Turano, M. A. Cremonini, C. Luchinat, and H. B. Gray, *Proteins* **29,** 68–76 (1997).
7. L.-F. Kao and V. J. Hruby, *J. Magn. Reson.* **70,** 394–407 (1986).
8. Z. Chen, J. S. de Ropp, G. Hernández, and G. N. La Mar, *J. Am. Chem. Soc.* **116,** 8772–8783 (1994).
9. (a) I. Bertini, A. Dikiy, C. Luchinat, M. Piccioli, and D. Tarchi, *J. Magn. Reson. B* **103,** 278–283 (1994); (b) L. Banci, W. Bermel, C. Luchinat, R. Pierattelli, and D. Tarchi, *Magn. Res. Chem.* **31,** S3–S7 (1993).
10. I. Bertini, F. Capozzi, S. Ciurli, C. Luchinat, L. Messori and M. Piccioli, *J. Am. Chem. Soc.* **114,** 3332–3340 (1992). [The use of MLEV-16 in front of a 2D sequence to "presaturate" the diamagnetic part of the spectrum has been reported but not discussed in the text]
11. T. Inubushi and E. D. Becker, *J. Magn. Reson.* **51,** 128–133 (1983).
12. M. Piccioli, *J. Magn. Reson. B* **110,** 202–204 (1996).
13. I. Morishima and S. Ogawa, *in* "Oxidases and Related Redox Systems" (T. E. King *et al.,* Eds.), Pergamon, Oxford/New York (1982).
14. (a) H. Goff, E. T. Shimomura, Y. J. Lee, and W. R. Scheidt, *Inorg. Chem.* **23,** 315–321 (1984); (b) R. D. Arasasingham, A. L. Balch, C. R. Cornman, J. S. de Ropp, K. Eguchi, and G. N. La Mar, *Inorg. Chem.* **29,** 1847–1850 (1990); (c) B. Garcia, C.-H. Lee, A. Blaskó, and T. C. Bruice, *J. Am. Chem. Soc.* **113,** 8118–8126 (1991).
15. (a) S. Adachi, S. Nagano, K. Ishimori, Y. Watanabe, I. Morishima, T. Egawa, T. Kitagawa, and R. Makino, *Biochemistry* **32,** 241–252 (1993); (b) D. P. Hildebrand, D. L. Burk, R. Maurus, J. C. Ferrer, G. D. Brayer, and A. G. Mauk, *Biochemistry* **34,** 1997–2005 (1995).
16. J. Stonehouse, P. Adell, J. Keller, and A. J. Shaka, *J. Am. Chem. Soc.* **116,** 6037–6038 (1994).
17. D. Marion and A. Bax, *J. Magn. Reson.* **79,** 352–356 (1988).
18. (a) G. N. La Mar, D. L. Budd, K. M. Smith, and K. C. Langry, *J. Am. Chem. Soc.* **102,** 1822–1827 (1980); (b) U. Pande, G. N. La Mar, J. T. J. Lecomte, F. Ascoli, M. Brunori, K. M. Smith, R. K. Pandey, D. W. Parish, and V. Thanabal, *Biochemistry* **25,** 5638–5646 (1986).