Journal of Magnetic Resonance **138,** 268–276 (1999)
Article ID jmre.1999.1735, available online at http://www.idealibrary.com on IDEAL®

Selective Injection of Magnetization by Slow Chemical Exchange in NMR

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Received December 1, 1998; revised February 8, 1999

In a system in slow dynamic equilibrium two NMR methods are shown to be suitable for injecting magnetization from one resonance to another by means of slow chemical exchange. The combined outputs of the methods may be employed to measure the value of the off-rate constant κ_{off} in the complex. The methods are implemented experimentally using the complex of molecules composed of the enzyme *Escherichia coli* dihydrofolate reductase (DHFR) and the ligand folate. In an equilibrium solution with DHFR, folate is known to undergo chemical exchange between a free state and a bound state. The modified synchronous nutation method is applied to a spin of the folate molecule in the free and bound states; magnetization transfer occurs between the two sites due to the underlying exchange process. As a preliminary step for the application of the synchronous nutation method, a new one-dimensional ^{1}H NMR technique is proposed which facilitates the assignment of the resonance of a spin in the bound state, provided the resonance of its exchange partner in the free state is known. This experiment is also used to obtain quantitative estimates of the transverse relaxation rate constant of the bound resonance. The numerical procedure necessary to analyze the experimental results of the synchronous nutation experiment is presented. © 1999 Academic Press

Key Words: **nuclear magnetic resonance (NMR); dihydrofolate reductase (DHFR); injection of magnetization; synchonous nutation.**

I. INTRODUCTION

A method allowing improvement in control over the dynamics of a nuclear spin system is inherently of interest for spin engineers. At the same time, novel NMR methods may provide insight in designing protocols for manipulation of different spin systems or other types of quantum networks by means of classical radiofrequency sources. NMR spectroscopy can play a role that goes beyond the invaluable technical one, which has proven so successful in characterizing the structure and dynamics of many small to medium-sized molecules or complexes of molecules in solution (*1, 2*). From this latter point of view, the measurement of exchange rate constants in systems in dynamic equilibrium by NMR has developed into an important tool for characterizing various biochemical processes, such as slow motions at particular locations within a molecule or the binding of a ligand to a receptor, where the ligand exchanges between a free state and a bound state. From the viewpoint expressed at the beginning of the paragraph, exchange processes in systems in dynamic equilibrium are a means by which a better understanding of the dynamics of nuclear spin systems can be gained, resulting in improved dynamic control which in turn augments the possible applications.

The modified synchronous nutation method was analyzed in some detail in the past (*3*): its main purpose is to isolate a pair of nonequivalent spins with respect to cross-relaxation or slow exchange involving other spins. Several other experimental schemes such as the original synchronous nutation method (*4*), MINSY (*5*), BD-NOESY (*6, 7*), QUIET-NOESY (*8*), and QUIET-EXSY (*9*) tend to achieve the same goals with, in many situations, an acceptable level of confidence. By using one of these methods the difficulties associated with the quantification of spin diffusion can be avoided. As a result, the analysis of experimental data can be performed with fewer preliminary assumptions, reducing the number of possible ambiguities in the derivation of quantitative values. The methods are not usually meant to produce a complete structural or dynamic study of a macromolecule but rather to focus on specific sites of particular interest. In this paper we do not want to compare these different methods or to stress which one would be more appropriate to employ in specific cases. This comparison can be made and the results will be published elsewhere. The goal of this paper is rather to present to the spin engineer the modified synchronous nutation method applied to a complex of molecules undergoing slow chemical exchange.

The modified synchronous nutation method is a one-dimensional experiment. Spectral overlap is overcome by incoherent processes (*10*). In terms of biochemical applications the modified synchronous method has already been used to expand the

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1090-7807/99 $30.00

possibilities of measuring a self-relaxation rate constant of a specific ^{1}H nuclear spin in crowded spectra (*10*), as well as to concentrate on the measurement of the cross-relaxation rate constant between two specific spins or the rate constant of a slow conformational exchange within a molecule (*11*).

In the present study the modified synchronous nutation method is applied, using chemical exchange as the underlying mechanism for the transfer of magnetization. During the mixing time the two sidebands of a radiofrequency field are placed on resonance with the different frequencies associated with a free resonance and the corresponding bound resonance of the ligand. It can be proven that the magnetization exchanged between the irradiated spins is free from magnetization transferred from other spins by cross-relaxation or slow exchange processes (*3*). As a result, taking into account only the parameters characterizing the pair of irradiated spins, we can give an estimate of the value of the off-rate constant κ_{off} of a ligand, which oscillates slowly on the NMR time scale between a free state and an enzyme-bound state. A preparatory ^{1}H NMR experiment utilizes the effects of the underlying exchange process to inject magnetization from the site of a nuclear spin in the free state to the site of the corresponding nuclear spin in the bound state of the ligand. If the resonance frequency of the spin in the free state is known, the resonance frequency of its bound partner can be assigned. This step allows one to avoid specific isotopic enrichment of either the ligand or the substrate. Moreover, subsequent manipulations of the spin which has received magnetization allow the measurement of the sum of the transverse self-relaxation rate constant of the bound resonance ρ^t_{bound} with the off-rate constant κ_{off}.

The NMR methods presented in this study are applied to the biomolecular complex composed of *Esherichia coli* dihydrofolate reductase (*E. coli* DHFR) and folate. *E. coli* DHFR is composed of 159 amino acid residues, has a molecular weight of 18 kDa, and has been characterized in exquisite mechanistic and structural detail (*12, 13*). The enzyme utilizes nicotinamide adenine dinucleotide phosphate (NADPH) to reduce 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F). DHFR also catalyzes the reduction of folate to H_4F, albeit less efficiently than the reduction of dihydrofolate. A detailed kinetic scheme is known (*14*), as are the energetic residues that interact with folate and NADPH (*15*). DHFR is necessary for maintaining intracellular pools of H_4F and its derivatives, which are essential cofactors for biosynthetic reactions requiring one-carbon unit transfer. Consequently, DHFR has proven to be an attractive target for rational drug design. DHFR is of mechanistic interest in the light of intriguing dynamic processes evident in the free enzyme and the enzyme/ligand complex (*16*). When present in solution together with the enzyme, the folate molecule is known to undergo slow chemical exchange on the NMR time scale between a free state and an enzyme-bound state. This biomolecular complex is therefore suitable for testing the spectroscopic methods presented in this paper.

II. MATERIALS AND METHODS

Purified *E. coli* DHFR was prepared as previously described (*16*). A 2 mM solution of *E. coli* DHFR and a 0.1 M solution of folate (Sigma Chemicals) were combined, yielding a binary *E. coli* DHFR–folate mixture in ^2H_2O solution, with a fourfold excess of folate. The pH of the solution was 6.8; it contained 0.05 M $KHPO_4$, 0.1 M KCl, and 0.001 M dithiothreitol. The sample was equilibrated for 15 min under argon at 295 K. All NMR spectra were acquired at 300 K on a Bruker AMX600 NMR spectrometer.

The modified synchronous nutation experiment necessitates precise knowledge of the frequencies of the two spins that are to be irradiated (*10, 11*). In a complex in dynamic equilibrium where one of the molecular species is of low molecular weight and undergoes chemical exchange between a bound and a free state, it is often the case that the nuclear spins belonging to the molecules in the free state give rise to sharp resonances in a conventional, one-dimensional ^{1}H NMR spectrum. Thus it is usually easy to find which "free" resonances are of interest for studying the exchange process. In contrast, the nuclear spins belonging to the molecule in the bound state have relaxation behavior similar to that of the nuclear spins of the macromolecule, and therefore their resonances are broadened and are not easily distinguishable from the resonances of the macromolecule.

In Fig. 1 a conventional, one-dimensional NMR spectrum of the binary mixture is presented. The free and bound peaks are indicated by arrows. Using natural abundance, i.e., with ^{12}C and ^{14}N folate, it was possible to identify the bound peak by the experiment presented in Fig. 2. This 1D ^{1}H NMR experiment starts with a selective 270° Gaussian pulse (*17*) of duration 3×10^{-2} s applied at the resonance frequency of the C_7H proton in the free state of folate. Immediately following this selective pulse, a nonselective 90° pulse is applied so that the C_7H spin associated with the free state is brought back parallel to the static magnetic field, while all the other resonances (in as far as they do not overlap with the free one) are brought to the transverse plane. Subsequently, a train of hard 180° pulses separated by a short duration, $\tau = 2 \times 10^{-3}$ s, is applied. During this period, the spin that was excited by the selective pulse at the beginning of the experiment and that is parallel to the Oz axis can exchange magnetization with its cross-relaxation or exchange partners. To bring the magnetization that is parallel to the Oz axis back to the transverse plane, a hard 90° pulse is applied. This pulse also has the effect of returning to the Oz axis the magnetization that was in the transverse plane during the train of nonselective 180° pulses. To obtain an estimate of the transverse relaxation of the spins to which magnetization was transferred during the train of 180° pulses, one can append to this pulse sequence a period (τ_m–180°–τ_m) and vary the delay τ_m. The 180° pulse in this optional period of relaxation is a selective or nonselective 180° pulse depending on whether the bound spin is scalarly coupled to other partners.

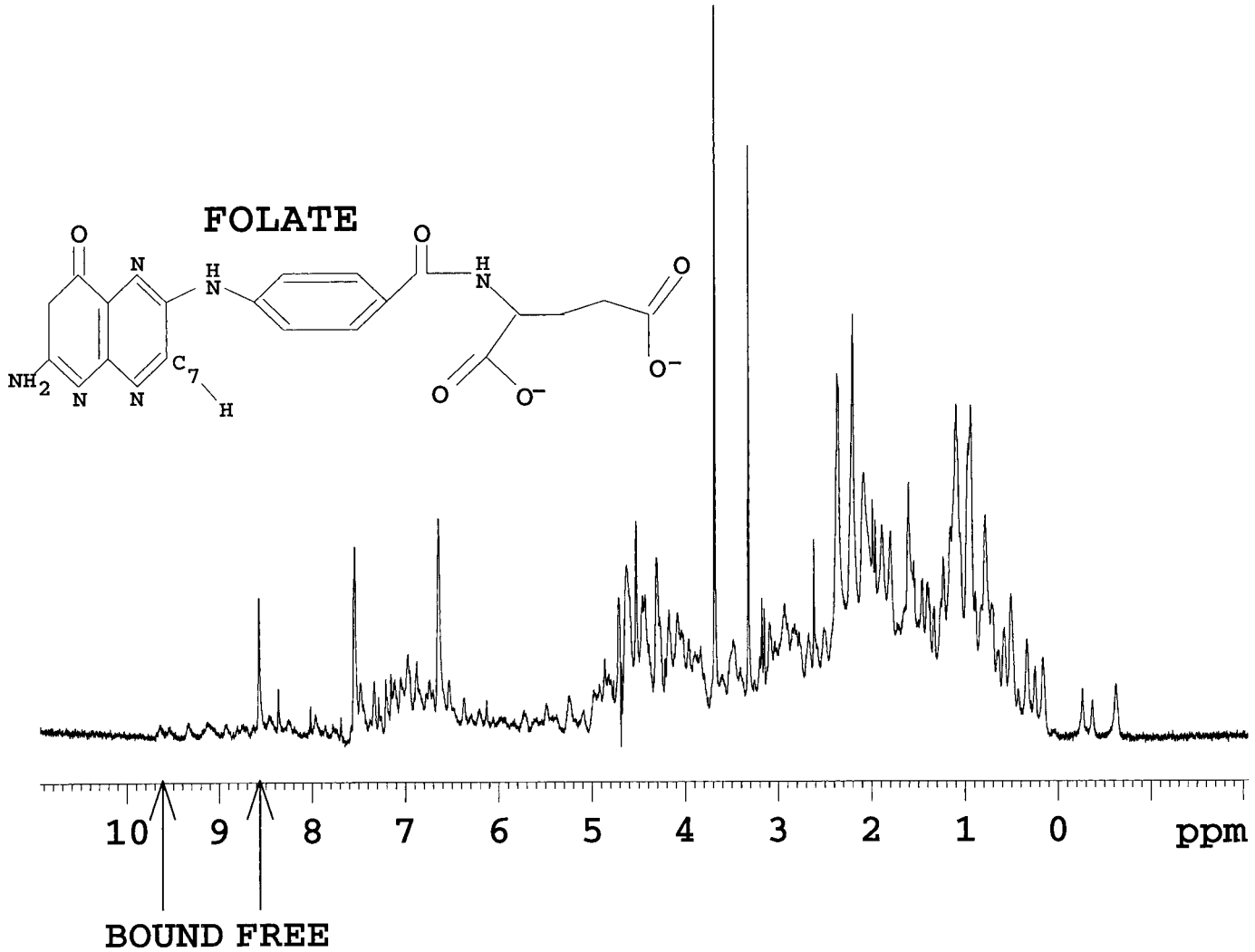


FIG. 1. Conventional ^{1}H NMR spectrum of the complex consisting of DHFR and folate. Eight transients were signal averaged. The bound and free resonances that are determined using the experiment presented in Fig. 2 are indicated by arrows. A drawing of the folate molecule is presented as an inset.

The decay curve obtained in this manner can generally be fitted to a single exponential function with a decay rate given by the sum of the transverse self-relaxation rate constant ρ^{t}_{bound} of the bound resonance and the off-rate constant κ_{off} of the exchange process. Application of this experiment to the *E. coli* DHFR–folate complex is presented in Fig. 2 for τ_m varying between 3×10^{-6} and 7.5×10^{-3} s. In each case 16 nonselective 180° pulses were applied; they were separated by 2×10^{-3} s, leading to a mixing time of 3.4×10^{-2} s for the longitudinal magnetization transfer period. The number of scans for each experiment was 1024. Each experiment was repeated three times to obtain an estimate of the experimental error.

The modified synchronous nutation method is applied to the *E. coli* DHFR–folate complex. The pulse sequence for this experiment is presented in the top panel of Fig. 3. The spin of the C_7H proton of the folate molecule in the free state is selectively rotated into the transverse plane by a 270° Gaussian pulse of 3×10^{-2} s duration. Immediately following this excitation pulse, an amplitude-modulated RF field is applied such that its two sidebands are coincident with the resonances of the spins of the C_7H proton in the free and bound states of the folate molecule. During this pulse the trajectories of the spins are best followed in the doubly rotating frame defined by the frequencies of the free and bound resonances. In this frame (neglecting the effect of off-resonance sidebands and assuming, as appropriate in our application, that there are no significant scalar J couplings present), each of the free and bound spins is forced to nutate synchronously in a plane perpendicular to the direction defined by the phase of the RF field. During this double-irradiation period the longitudinal components of the two irradiated spins exchange magnetization as in the laboratory frame, as a consequence of the chemical exchange the folate molecule undergoes. Since the double-irradiation period is always chosen to be greater than the inverse of the

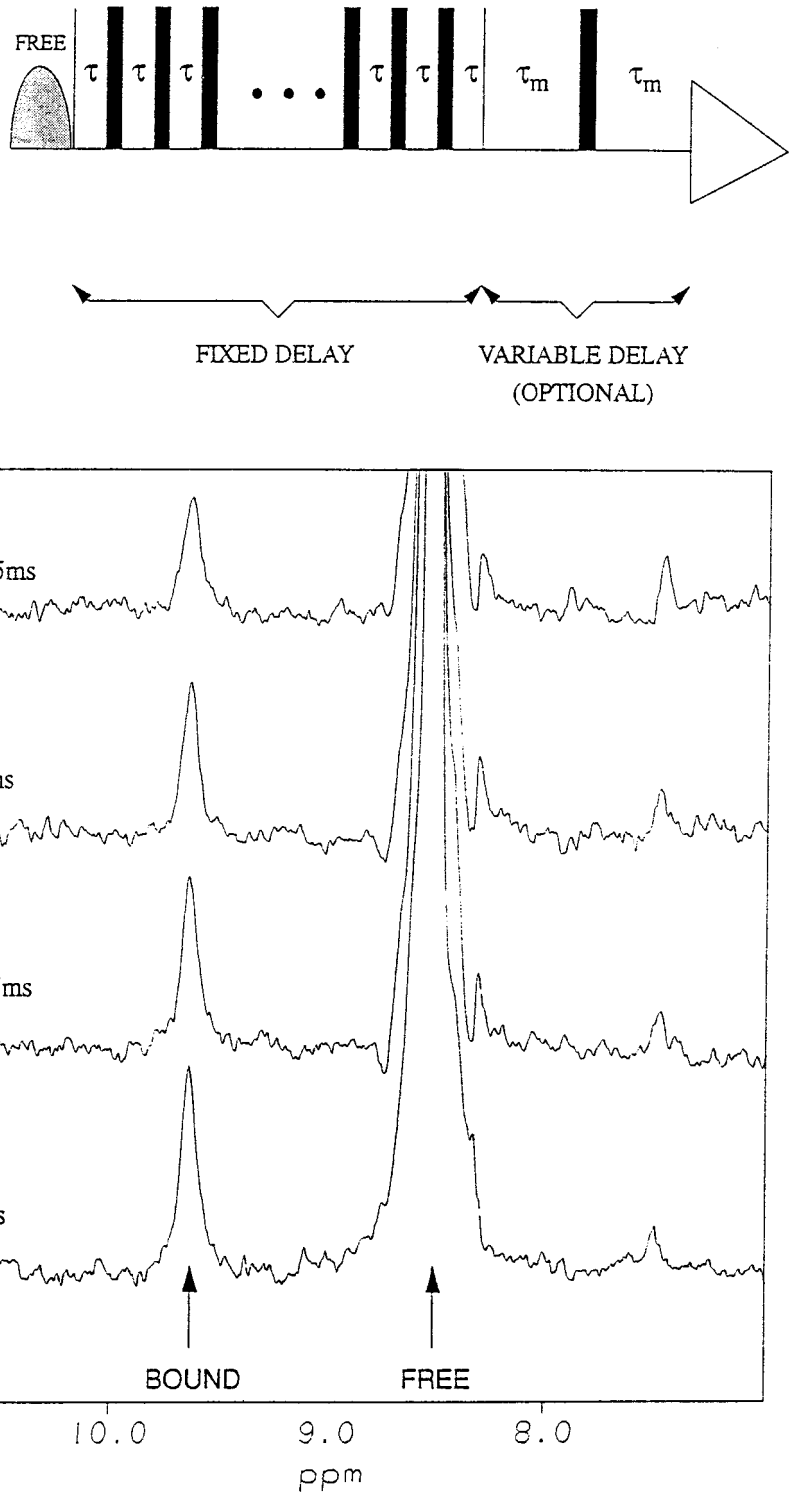
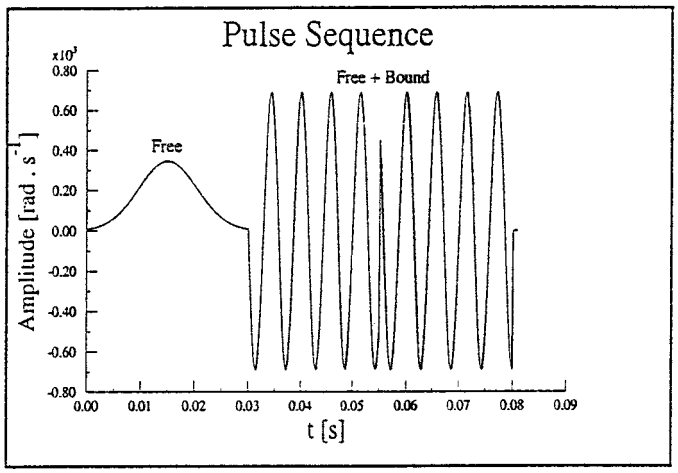


FIG. 2. (Top) Pulse sequence used to determine the resonance frequency of the bound resonance, knowing the frequency of the free resonance. The pulse sequence starts with a selective Gaussian 270° pulse, directly followed by a 90° nonselective pulse. After a delay τ a train of nonselective 180° pulses is applied, each of which is followed by a delay τ. A final 90° nonselective pulse is then applied, which has the same phase as the first one. Optionally, a period τ_m–180°–τ_m is applied to measure the transverse relaxation of the spin to which magnetization was injected. (Bottom) Results of the experiment (including the optional period) as applied to the complex DHFR–folate. The selective pulse is applied at the resonance frequency of the spin of the C_7H proton in the free folate. The delay τ_m is varied between 3×10^{-6} and 7.5×10^{-3} s, and its value is indicated beside each spectrum. In each case sixteen 180° nonselective pulses are applied. They are separated by 2×10^{-3} s leading to a longitudinal mixing time of 34×10^{-3} s. The number of scans for each experiment is 1024.



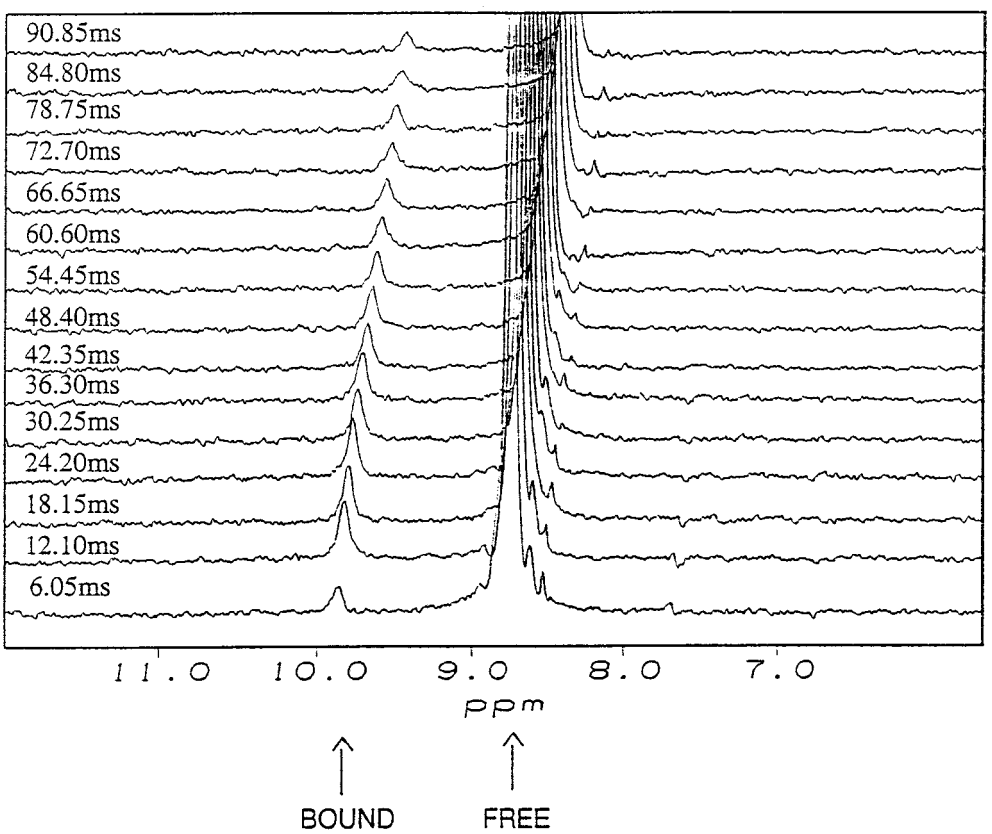


FIG. 3. (Top) The pulse sequence for the modified synchronous experiment as applied to the complex DHFR–folate is presented. It starts with a selective Gaussian 270° pulse on resonance with the spin of the C_7H proton in the free folate (free spin). Immediately following this pulse an amplitude modulated RF field is applied whose sidebands are coincident with the resonance frequencies of the spins of the C_7H protons in the free and bound states of the ligand (free and bound spins). (Bottom) Results obtained for the modified synchronous nutation experiment when the duration of the double-irradiation field is varied between 6.05×10^{-3} and 90.85×10^{-3} s in steps of 6.05×10^{-3} s. The duration of the double-irradiation RF field is indicated beside each spectrum. The number of scans for each experiment is 2048.

frequency difference between the two spins, the transfer of transverse magnetization between the two spins is averaged to zero. In the bottom panel of Fig. 3, 15 experiments are presented which correspond to a variation of the duration of the amplitude-modulated RF field between 6.05×10^{-3} and 90.85×10^{-3} s in steps of 6.05×10^{-3} s. The number of scans

for each 1D experiment is 2048. Each experiment was repeated three times to obtain an estimation of the experimental error.

To fit the experimental data, numerical simulations for a three-spin system are performed, employing a computer simulation program whose supporting mathematical formalism was described earlier (*18*). For the numerical simulations it is assumed that the first and second spins are spatially close to each other while the third spin is spatially far away from the two other spins. In this manner, the relaxation behavior of the spins in the different states of the ligand can be mimicked. Spin one and spin three are allowed to exchange magnetization via the exchange process. In our application to the complex consisting of *E. coli* DHFR and folate, spin one (bound state of folate) and spin three (free state of folate) are the spins of the C_7H proton of the folate molecule that exchange their frequencies during the exchange process, while spin two represents a spin of the substrate or possibly another spin of the folate molecule in the bound state. Its presence is necessary to ensure that spin one will have the proper self-relaxation rate constants. A simple exponential damping of the transverse component of spin three is used to match the transverse relaxation rate constant that was measured experimentally for the spin of the C_7H proton of the folate molecule in D_2O solution.

III. RESULTS AND DISCUSSIONS

The experiment presented in the top panel of Fig. 2 is similar to a one-dimensional NOESY difference experiment, with an initial selective excitation. The advantage in the present case, however, is to perform the difference in a single experiment. This experiment is also similar to the water filter experiments that were proposed to study solvent-exchangeable protons in biomolecules (*19*). We, however, utilize a train of 180° pulses during the mixing time, which has the effect of preventing the spins not affected by the selective pulse from relaxing to equilibrium, as will be demonstrated below. To study solvent-exchangeable protons with the method presented here would necessitate to analyze carefully the effects of radiation damping during the train of 180° pulses. Our experiment separates, along perpendicular axes, spins that are in the spin diffusion network of the spin selectively excited at the beginning of the experiment and the remaining spins. It thus allows the subsequent manipulation of these two types of spins to be accomplished independently. For example, the bottom panel of Fig. 2 shows the results obtained when the bound spin, to which magnetization was transferred during the first part of the experiment, is allowed to relax in the transverse plane for variable amounts of time. From these experiments an estimate of the sum $\rho^{t}_{\text{bound}} + \kappa_{\text{off}}$ is found to be $73.0 \pm 6.2\ s^{-1}$.

If a spin δ is not contained in the spin diffusion network of the spin which is selectively excited at the beginning of the experiment, the amount of longitudinal magnetization present on this spin just prior to the last nonselective 90° pulse can be made negligible by a proper choice of the interval τ. For the spin δ we have, after the first two pulses (selective 270° and nonselective 90°),

$$\langle I_{\delta z}\rangle(0) - \langle I_{\delta z}\rangle^{\text{eq}} \neq 0, \qquad [1]$$

where $\langle I_{\delta z}\rangle^{\text{eq}}$ is the equilibrium magnetization on spin δ. It is then possible that some amount of magnetization will appear along the Oz axis due to the recovery of this longitudinal component. At the end of the first τ period we have

$$\langle I_{\delta z}\rangle(\tau) = \langle I_{\delta z}\rangle^{\text{eq}}(1 - \exp(-\rho_\delta \tau)), \qquad [2]$$

where ρ_δ is the longitudinal self-relaxation rate constant of the spin δ. Negating this value to insert it as the initial condition for the second τ period just after the first 180° pulse, we obtain at the end of the second τ period

$$\langle I_{\delta z}\rangle(2\tau) = \langle I_{\delta z}\rangle^{\text{eq}}(1 - (2 - \exp(-\rho_\delta \tau))\exp(-\rho_\delta \tau)) \qquad [3]$$

$$= \langle I_{\delta z}\rangle^{\text{eq}}(1 - 2\exp(-\rho_\delta \tau) + \exp(-2\rho_\delta \tau)). \qquad [4]$$

Pursuing this procedure until the end of the last τ period after the last (Nth, N a fixed integer) 180° pulse we obtain

$$\langle I_{\delta z}\rangle((N+1)\tau) = \langle I_{\delta z}\rangle^{\text{eq}}\Big(1 + 2\sum_{n=1}^{N}(-1)^n \exp(-n\rho_\delta \tau) + (-1)^{N+1}\exp(-(N+1)\rho_\delta \tau)\Big). \qquad [5]$$

If we choose τ small enough so that $\rho_\delta \tau \ll 1$ and we develop the exponentials in the equation above in Taylor series around zero, up to the first order we obtain

$$\langle I_{\delta z}\rangle((N+1)\tau) \approx \langle I_{\delta z}\rangle^{\text{eq}}\Big[1 + (-1)^{N+1} + 2\sum_{n=1}^{N}(-1)^n + 2\sum_{n=1}^{N}(-1)^n(-n\rho_\delta \tau) - (-1)^{N+1}((N+1)\rho_\delta \tau)\Big]. \qquad [6]$$

Simplifying the above expression we derive

$$\langle I_{\delta z}\rangle((N+1)\tau) \approx -\rho_\delta \tau \langle I_{\delta z}\rangle^{\text{eq}}\Big[2\sum_{n=1}^{N}(-1)^n n + (-1)^{N+1}(N+1)\Big]. \qquad [7]$$

The value of the term within square brackets in the expression above is equal to 1 if N is even and to -2 if N is odd. Therefore, by choosing τ small enough, $\langle I_{\delta z}\rangle$ $((N+1)\tau)$

remains negligible. It should be noted that the replacement of the train of 180° pulses by some gradient pulse to wipe out any transverse magnetization will not prevent longitudinal magnetization from recovering during the mixing time.

Numerous methods exist to measure the off-rate constant of a slow chemical process with, in many cases, sufficient accuracy (*20–22*). However, in situations where the off-rate constant turns out to be of the same order as the cross- or self-relaxation rate constants, it is important to quantify the amount of spin diffusion generated in the system. In this manner the value derived for the off-rate constant can be made more precise. It is thus of interest to know that methods such as synchronous nutation can suppress the effect of spin diffusion early in the experimental stage and even for long mixing times. To calculate the off-rate constant, values of the spectral density function characterizing the molecular motions have still to be obtained, but only locally, at the site of the two chosen spins. The self-relaxation rate constants of the two spins involved need to be known, but not those of the spins belonging to their spin diffusion network. The number of parameters for analysis of the experimental data is therefore greatly reduced. Experimental results are discussed by means of the parameters characterizing a two-spin system. Quantitative values of rate processes can be derived without proposing a solution for the entire structural and dynamic problem.

In the experimental spectra shown in the bottom panel of Fig. 3, only the free and bound resonances are present, proving the selectivity of the transfer of magnetization. With a molecule the size of DHFR, it seems quite remarkable to obtain some intensity for the bound resonance after 0.0985 s of double irradiation, during which the spins affected by the RF field spent part of the time close to or in the transverse plane. However, this intensity comes from the sustained flow of magnetization between the free and the bound spins that takes place during the double-irradiation period. The numerical simulation program used to analyze the set of experiments can currently handle three spins, in principle belonging to a molecular system with a single correlation time. Lengths and orientations of the internuclear vectors are given. Only dipole–dipole interactions are taken into account to describe relaxation. We performed simulations using a Lorentzian spectral density function. The distance between spin one (bound spin) and spin two (representing a spin of DHFR or another spin of folate in the bound state) was varied for each variation of the values of the parameter characterizing the exchange between spin one and spin three (free spin). It was necessary to add an exponential damping term to the transverse relaxation of spin three as, for reasons that need to be explored further, the experimentally measured transverse self-relaxation rate constant did not lead to the expected ratio when divided by the corresponding longitudinal self-relaxation rate constant. With this procedure, the best fit for the experiment can be accounted for by an off-rate constant in the range $59\ s^{-1} < \kappa_{off} < 67\ s^{-1}$ and a transverse self-relaxation rate constant in the range $2.48\ s^{-1} < \rho^{t}_{bound} < 18.69\ s^{-1}$. A large range of values for κ_{off} and ρ^{t}_{bound} in these intervals are compatible with the results given by the other experiment presented in this paper, which gave $66.8\ s^{-1} < \kappa_{off} + \rho^{t}_{bound} < 79.2\ s^{-1}$. As mentioned above, ρ^{t}_{free} for the spin of the ^{1}H nucleus of interest was measured in a sample containing only the folate molecule. In this case the value of the self-relaxation rate constant was found to be $\rho^{t}_{free} = 9.3 \pm 0.5\ s^{-1}$, higher than the lower bound for ρ^{t}_{bound} of $2.48\ s^{-1}$. Taking the value of $9.3\ s^{-1}$ as the lower bound for ρ^{t}_{bound}, the values allowed by the numerical fit for κ_{off} are $59\ s^{-1} < \kappa_{off} < 66\ s^{-1}$. The values contained in this interval are slightly higher than the value of $35 \pm 12\ s^{-1}$ obtained by competition experiments (*23*). Figure 4 superimposes the experimental data points (with their error bars) with the points obtained from one of the representative simulations leading to the best fit. The lines joining the points are included for visual assistance. Except for $\tau_{dir} = 0.0125 \times 10^{-3}$ s, the simulated points lie close to or within the error intervals of their associated experimental points. If the experimental error were represented by the mean of the particular experimental errors shown on the graph, 14 of the 16 points shown could be fitted. The remaining discrepancies can probably be explained by the use in the fitting procedure of a simple motional model. Moreover, the unusual value of the ratio $\rho^{t}_{free}/\rho^{l}_{free}$ is indicative of some supplementary relaxation mechanism that may exist in the bound state of the folate molecule and cannot be taken into account in the simulation until more information about this process can be obtained. It might be said in this particular situation that the value of $59\ s^{-1}$ obtained for κ_{off} is already well above the value of any self- or cross-relaxation rate constants in the molecular system, thus rendering negligible the contribution of spin diffusion to the sum of the longitudinal self-relaxation rate constant and off-rate constant that could be obtained by a normal inversion–recovery experiment. This remark is true when we have in mind only the biochemical application. However, it is now demonstrated that the modified synchronous nutation method can be implemented in systems in slow dynamic equilibrium. In the past we have already proven (*3*) that the synchronous nutation method would perform a very good isolation with respect to cross-relaxation or slow exchange involving other spins, even for very slow rate constants and long mixing times. As a result of the combined effects of these remarks the method should become attractive for use in specific situations where the off-rate constant is of the same order as the relaxation rate constants.

IV. CONCLUSION

In this paper we have displayed some inherent advantages offered by the modified synchronous nutation method. Using this method we have demonstrated that it is possible to control the selective injection of magnetization from one nuclear spin frequency to another by a slow chemical exchange process assumed to be active in the associated molecular system. The

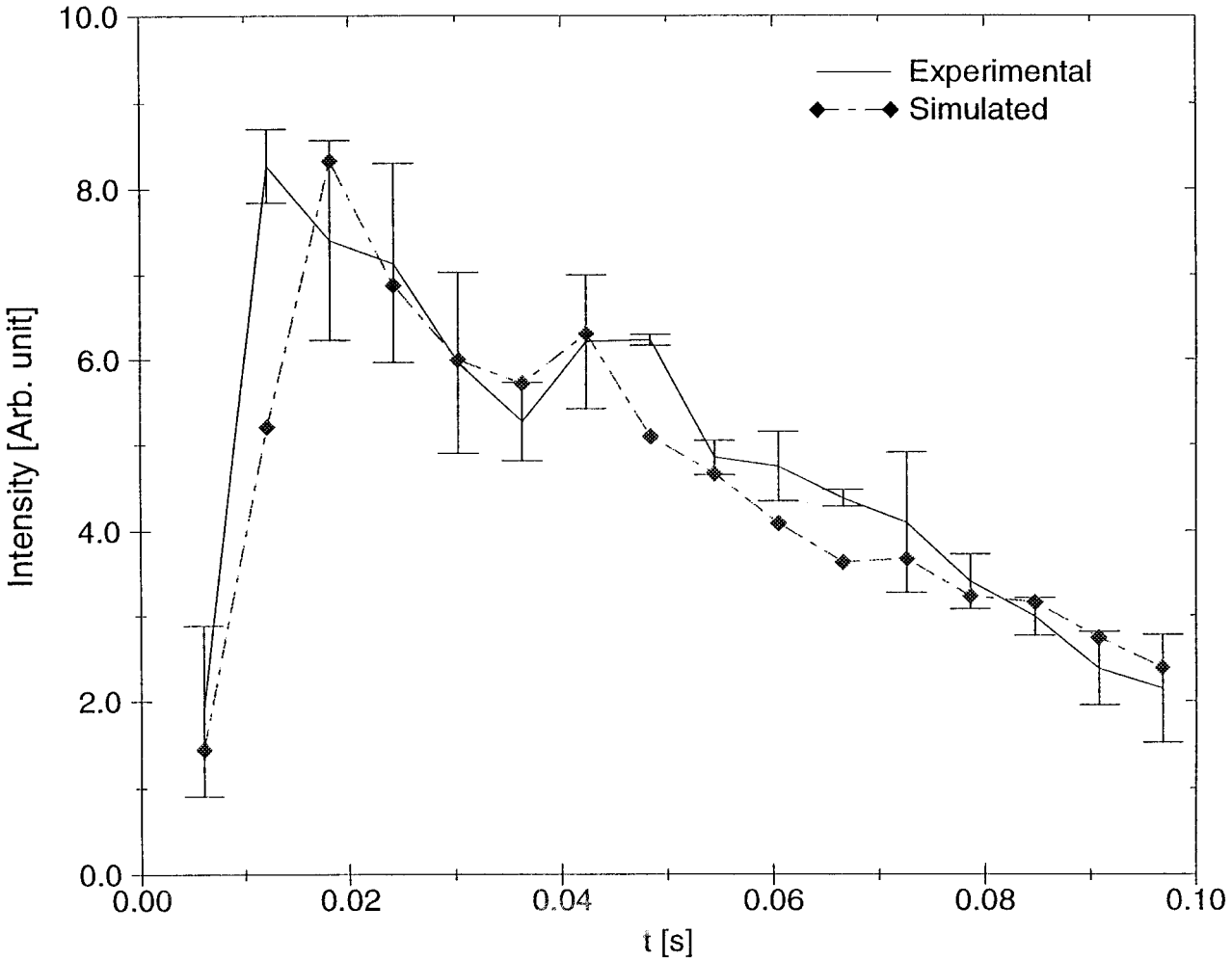


FIG. 4. Comparison of the experimental values obtained with the values derived by numerical simulations of the experiments. The points obtained from one of the representative simulations leading to the best fit are plotted. Lines joining the points are drawn for visual assistance.

latter is composed of a substrate and a ligand, binding and dissociating according to a time constant which is slow on the NMR time scale. The selectivity in the injection of magnetization resulted from the proper constraints imposed on the evolution of the spin system by the application of the modified synchronous nutation method. A preliminary one-dimensional ^{1}H NMR experiment was introduced to permit the determination of the resonance frequency of the spin attached to the nucleus of the active site of the ligand molecule in the bound state. This experiment was also used to obtain a quantitative estimate of the sum of the off-rate constant and the transverse self-relaxation rate constant of this spin. The modified synchronous nutation experiment was employed to obtain, as a function of the double-irradiation duration (mixing time), the amount of magnetization transferred to the bound resonance from the free resonance by the oscillatory binding process. In the synchronous nutation experiment these values were not affected by spin diffusion processes. As a consequence, the multiparameter fit that was performed to analyze the experimental data did not need to take into account spin diffusion. Application in the complex of molecules in slow dynamic equilibrium, consisting of *E. coli* DHFR and folate, was presented. By combining the results of the demonstration of the selective injection of magnetization by means of a chemical exchange process and the experimental evidence of feasibility given by the application to the DHFR–folate complex, we have shown that in specific situations where the off-rate constant would be of the same order as the relaxation rate constants a greater precision for the value of the off-rate constant could be derived by this method.

ACKNOWLEDGMENTS

We are indebted to Dr. P. E. Wright for comments and suggestions during the course of the project and to Dr. G. Bodenhausen for a careful reading of an early version of the manuscript. This work was supported by the National Institutes of Health (GM40089). B.B. acknowledges the Swiss National Foundation (Grant 823A-033368), the Center for Interdisciplinary Magnetic Resonance at the National High Magnetic Field Laboratory, and The Beckman

Research Institute at The National Medical Center of the City of Hope, through which part of this work was supported. D.M.E. acknowledges a postdoctoral fellowship from the American Cancer Society. Most of the numerical simulations presented in this work were performed at the National High Magnetic Field Laboratory.

REFERENCES

1. K. Wüthrich, "NMR of Proteins and Nucleic Acids," Wiley, New York (1986).
2. J. Cavanagh, W. Fairbrother, A. G. Palmer III, and N. Skelton, "Protein NMR Spectroscopy, Principles and Practices," Academic Press, San Diego (1996).
3. B. Boulat, I. Najfeld, and M. Rance, *J. Magn. Reson. A* **120,** 223–230 (1996).
4. I. Burghardt, R. Konrat, B. Boulat, S. J. F. Vincent, and G. Bodenhausen, *J. Chem. Phys.* **98,** 1721–1736 (1993).
5. W. Massefski, Jr., and A. G. Redfield, *J. Magn. Reson.* **78,** 150–153 (1988).
6. J. Fejzo, W. M. Westler, S. Macura, and J. L. Markley, *J. Am. Chem. Soc.* **112,** 2574–2577 (1990).
7. J. Fejzo, W. M. Westler, S. Macura, and J. L. Markely, *J. Am. Chem. Soc.* **114,** 1523–1524 (1992).
8. C. Zwahlen, S. J. F. Vincent, L. Di Bari, M. H. Levitt, and G. Bodenhausen, *J. Am. Chem. Soc.* **116,** 362–368 (1994).
9. C. Zwahlen, S. J. F. Vincent, M. Schwager, and G. Bodenhausen, *Chem. Eur. J.* **116,** 45–49 (1996).
10. B. Boulat and M. Rance, *J. Magn. Reson. B* **110,** 288–297 (1996).
11. B. Boulat and M. Rance, *J. Chem. Phys.* **101,** 7273–7282 (1994).
12. C. Bystroff, S. J. Oatly, and J. Kraut, *Biochemistry* **30,** 3263–3277 (1990).
13. C. J. Falzone, J. Cavanagh, M. Cowart, A. G. Palmer III, C. R. Matthews, S. J. Benkovic, and P. E. Wright, *J. Biomol. NMR* **4,** 349–366 (1994).
14. C. A. Fierke, K. A. Johnson, and S. J. Benkovic, *Biochemistry* **26,** 4085–4092 (1987).
15. C. Bystroff and J. Kraut, *Biochemistry* **30,** 2227–2239 (1991).
16. D. M. Epstein, S. J. Benkovic, and P. E. Wright, *Biochemistry* **34,** 11037–11048 (1995).
17. L. Emsley and G. Bodenhausen, *J. Magn. Reson.* **82,** 211 (1989).
18. B. Boulat and M. Rance, *Mol. Phys.* **83,** 1021–1039 (1994).
19. S. Mori, C. Abeygunawarandana, P. C. M. Van Zil, and J. M. Berg, *J. Magn. Reson. B* **110,** 96–101 (1996).
20. A. Abragam, "Principles of Magnetic Resonance," Clarendon, Oxford (1961).
21. J. I. Kaplan and G. Fraenkel, "NMR of Chemically Exchanging Spins," Academic Press, New York, (1980).
22. R. R. Ernst, G. Bodenhausen, and A. Wokaun, "Principles of Nuclear Magnetic Resonance in One and Two Dimensions," Clarendon, Oxford (1987).
23. B. A. Posner, L. Li, R. Bethell, T. Tsuji, and S. J. Benkovic, *Biochemistry* **35,** 1653–1663 (1996).