Buildup Rates of the Nuclear Overhauser Effect Measured by Two-Dimensional Proton Magnetic Resonance Spectroscopy: Implications for Studies of Protein Conformation

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Abstract: It is demonstrated, by means of experiments with the basic pancreatic trypsin inhibitor, that the buildup rates of the nuclear Overhauser effect can be measured by two-dimensional NMR spectroscopy. Qualitative correlations between the buildup rates of first-order Overhauser effects, which arise from direct dipole–dipole coupling between closely spaced protons, and the proton–proton distances in the protein conformation are established. Second-order Overhauser effects due to spin diffusion by cross-relaxation between more distant protons are also identified. On the basis of these observations, potentialities and limitations of two-dimensional nuclear Overhauser enhancement spectroscopy for studies of the conformations of biological macromolecules are discussed and suggestions made for improved experimental procedures. For quantitative measurements of Overhauser effects, the use of phase-sensitive spectra and of techniques for selective suppression of J cross-peaks in data sets recorded with very short mixing times appears particularly important.

The nuclear Overhauser effect (NOE)[2] is the fractional change in intensity by cross-relaxation of one NMR line when another resonance is perturbed. In the presence of fast motional processes, the steady-state NOE is a sensitive measure of the distance between observed and perturbed nuclei, and it has long been a valuable tool for structural studies of small molecules.[3] In macromolecules at high magnetic fields, however, spin diffusion can become quite efficient,[4,5] causing the conventional steady-state NOE's[3] to be less specific and hence less useful. In contrast, the initial buildup rates of NOE's in macromolecular systems are simply related to the inverse sixth power of the distance between the observed and the presaturated proton.[3-7] One-dimensional experiments for measurements of NOE buildup rates in macromolecules have been developed.[5,6,8] Their practical use is limited, however, since they require long accumulation times and because of the poor selectivity for preirradiation of individual resonance lines in crowded regions of the one-dimensional ^{1}H NMR spectra. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) is a more powerful and more efficient method for studies of selective NOE's between neighboring protons in the spatial structures of biological macromolecules. With a single instrument setting, NOESY can provide a complete set of proton–proton Overhauser effects in a protein.[9] For biological work it is further of particular interest that NOESY spectra can be recorded essentially as easily in H_2O solution as in deuterated solvents.[10] Overall, with the use of two-dimensional (2D) spectroscopy, measurements of proton–proton NOE's should become a generally applicable approach for the determination of the molecular conformations of biological macromolecules.[11-14] So far important information on protein conformations was obtained from semiquantitative interpretations of NOESY data.[10,12,14,15] Futher refinement of the spatial structures will depend on more quantitative interpretations of the NOE's. As a first step in this direction, the present paper reports on measurements of τ_m-dependent features in NOESY spectra of the basic pancreatic trypsin inhibitor (BPTI), a small globular protein.

Methods and Experimental Procedures

Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY)[9] uses a recently developed 2D NMR method for investigations of cross-relaxation and chemical exchange processes.[16] As shown in the experimental scheme of Figure 1A, the experiment consists of a sequence of three nonselective 90° pulses. During the evolution period between the first and the second pulse, t_1, the various magnetization components are frequency-labeled to mark their origin. During the mixing period between the second and the third pulse, τ_m, selective homonuclear NOE's between different protons are building up by cross-relaxation through mutual dipolar interactions. The signal is recorded immediately after the third pulse as a function of t_2. In accordance with the general 2D spectroscopy principle,[17] the experiment for fixed τ_m is repeated for a set of equidistant t_1 values. A two-dimensional Fourier transformation of the data matrix $s(t_1,t_2;\tau_m)$ then produces the desired frequency domain spectrum, $S(\omega_1,\omega_2;\tau_m)$ (Figure 1B). In the NOESY spectrum one observes peaks on the diagonal which disects the two frequency axes (Figure 1B). These peaks correspond to magnetization components which do not exchange with other components during the mixing time τ_m, and hence have the same frequencies during t_1 and t_2. Off-diagonal cross-peaks

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(2) Abbreviations used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; 2D, two-dimensional; δ, chemical shift; ppm, parts per million; J, spin–spin coupling constant in Hz; FID, free induction decay; BPTI, basic pancreatic trypsin inhibitor (Trasylol, Bayer A.G., Leverkusen).
(3) Noggle, J. H.; Schirmer, R. E. "The Nuclear Overhauser Effect"; Academic Press: New York, 1971.
(4) Kalk, A.; Berendsen, H. J. C. *J. Magn. Reson.* **1976**, *24*, 343-366. Hull, W. E.; Sykes, B. D. *J. Chem. Phys.* **1975**, *63*, 867-880.
(5) Gordon, S. L.; Wüthrich, K. *J. Am. Chem. Soc.* **1978**, *100*, 7094-7096.
(6) Wagner, G.; Wüthrich, K. *J. Magn. Reson.* **1979**, 33, 675-680.
(7) Bothner-By, A. A.; Noggle, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 5152-5155.
(8) Johnston, P. D.; Redfield, A. G. *Nucleic Acid Res.* **1978**, *5*, 3913-3927. Krishna, N. R.; Agresti, D. G.; Glickson, J. D.; Walter, R. *Biophys. J.* **1978**, *24*, 791-814.
(9) Kumar, A.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1-6.
(10) Kumar, A.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1156-1163.
(11) Bothner-By, A. A. In "Magnetic Resonance Studies in Biology"; Shulman, R. G., Ed.; Academic Press: New York, 1979; pp 177-219.
(12) Wagner, G.; Kumar, A.; Wüthrich, K. *Eur. J. Biochem.* **1981**, *114*, 375-384.
(13) Wüthrich, K.; Bösch, C.; Brown, L. R. *Biochem. Biophys. Res. Commun.* **1980**, 95, 1504-1509.
(14) Braun, W.; Bösch, C.; Brown, L. R.; Go, N.; Wüthrich, K. *Biochim. Biophys. Acta* **1981**, *667*, 377-396.
(15) Bösch, C.; Kumar, A.; Baumann, R.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.* **1981**, *42*, 159-163.
(16) (a) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546-4553. (b) Meier, B. H.; Ernst, R. R. *J. Am. Chem. Soc.* **1979**, *101*, 6441-6447. (c) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95-117.
(17) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229-2246.

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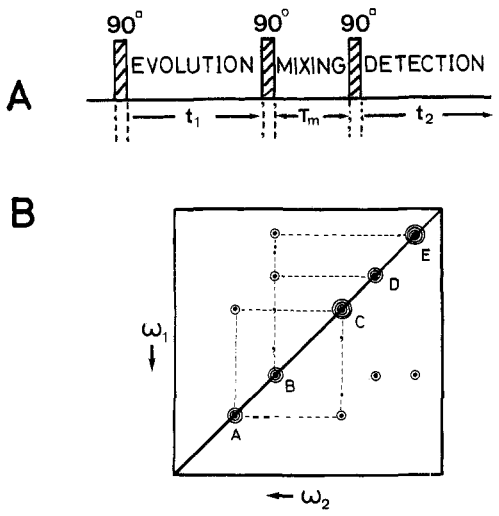

Figure 1. (A) Experimental scheme for two-dimensional nuclear Overhasuer enhancement spectroscopy (NOESY), which consists of a sequence of three nonselective 90° pulses. The pulses are separated by the evolution period, t_1, and the mixing period, τ_m, respectively. Immediately after the third pulse, the signal $s(t_1,t_2;\tau_m)$ is recorded. (B) Contour plot of a schematic NOESY spectrum. Among the five resonance lines A–E, NOE's are manifested by the cross-peaks between A and C, B and D, and B and E.

manifest exchange of magnetization. The magnetization transfer is due to dipole–dipole cross-relaxation during the mixing time. In the schematic NOESY spectrum of Figure 1B, peak A is dipole–dipole coupled with peak C, and peak B with D and E. Two sets of cross-peaks appear in symmetrical locations with respect to the diagonal peaks. Similar phenomena occur also in the presence of chemical exchange.[16a,b]

Fundamentally the physical situation in a NOESY experiment is similar to that in one-dimensional transient NOE experiments.[5] In both techniques the exchange of magnetization is initiated by a short radiofrequency pulse, and no radiofrequency field is applied while the NOE's are building up. In analogy to the transient NOE experiment,[5] the time development of NOE's in NOESY can be investigated when different two-dimensional spectra are recorded with different mixing times τ_m. For each τ_m value a complete set of NOE's is obtained. For work with macromolecules, NOESY is therefore much more efficient than one-dimensional NOE experiments.[9] Another important advantage is that NOESY uses *nonselective* pulses (Figure 1A) to obtain selective NOE's, whereas the selectivity of one-dimensional transient NOE experiments depends critically on the selectivity of the presaturation pulse.[5]

For the experiments in this paper we used a 0.02 M solution of the basic pancreatic trypsin inhibitor (BPTI) in ^2H_2O, p^{2}H 4.6. BPTI (Trasylol®) was a gift from the Farbenfabriken Bayer A.G., Leverkusen, F.R.G.; 360-MHz ^{1}H NMR spectra were recorded on a Bruker HX 360 spectrometer equipped with an Aspect 2000 data system. The previously developed software for handling of the large data matrices obtained in 2D experiments with biological macromolecules[18,19] was used. NOESY spectra were recorded using quadrature detection in both dimensions, with the carrier frequency at one end of the spectrum. Transverse components at the beginning of the mixing period and the axial peaks at $\omega_1 = 0$ were cancelled by addition of groups of 16 experiments with different phases. These phase cycles will be described in detail elsewhere. Absolute value spectra are presented either as stacked plots providing a three-dimensional view of the spectra, contour plots, or by means of cross sectins.[19,20] Further improvement of the 2D spectra can be obtained by triangular multiplication,[21] a technique based on the symmetry of the cross-peak pattern with respect to the diagonal peaks.

Results

Figure 2A shows a NOESY spectrum of BPTI which was recorded with a mixing time of 300 ms. On the diagonal from

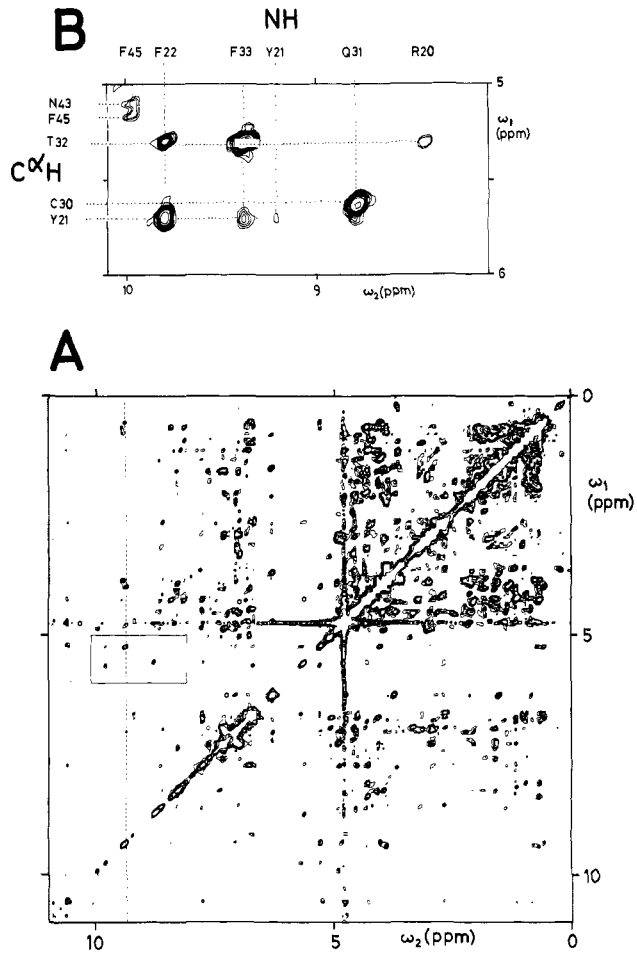

Figure 2. (A) Contour plot of a proton NOESY spectrum at 360 MHz of a 0.02 M solution of the basic pancreatic trypsin inhibitor (BPTI) in ^2H_2O, T = 24 °C, p^{2}H 4.6. The mixing time, τ_m, was 300 ms. The spectral width was 4000 Hz. The data set consisted of 512 points in both dimensions, 48 free induction decays were accumulated for each value of t_1, and the total accumulation time was 18 h. Before Fourier transformation the free induction decays were multiplied with a phase-shifted sine bell, $\sin[\pi(t + t_0)/t_s]$, where t_s was the experimental acquisition time and t_0/t_s was 1/64.[30] After Fourier transformation the spectrum was further improved by triangular multiplication.[21] An absolute value plot is shown. The vertical and horizontal spikes at 4.8 ppm are due to the resonance of the residual solvent protons. The dotted, vertical line at 9.39 ppm inidicates where the cross sections of Figure 4 were taken. (B) Plot on an expanded scale of the spectral region from 5.0 to 6.0 ppm in ω_1 and 8.1 to 10.1 ppm in ω_2, which is indicated by a solid rectangle in spectrum A. Lower contour levels were plotted than in A, so that additional peaks appear in spectrum B. The cross peaks are identified by the IUPAC-IUB one-letter symbols for amino acids and the position in the amino acid sequence of the C^α and amide protons.

the upper right to the lower left corner of the figure one recognizes a pattern of peaks which corresponds to that in the normal, one-dimensional ^{1}H NMR spectrum of BPTI.[22] Many of these resonances were previously individually assigned.[22,23] At 4.8 ppm there are a horizontal band and a vertical band of spurious noise which correspond to "tails" of the strong diagonal peak of the residual water protons. All other off-diagonal peaks manifest NOE's between resonances with corresponding frequencies ω_1 and ω_2 (see Figure 1). Obviously, a large number of selective NOE's can be observed in this spectrum. Many of these have previously been assigned to specific pairs of protons in the protein.[9,10,12,22,24,25]

(18) Nagayama, K.; Wüthrich, K.; Bachmann, P.; Ernst, R. R. *Biochem. Biophys. Res. Commun.* **1977**, *78*, 99–105.
(19) Nagayama, K.; Bachmann, P.; Wüthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1978**, *31*, 133–148.
(20) Nagayama, K.; Kumar, A.; Wüthrich, K; Ernst, R. R. *J. Magn. Reson.* **1980**, *40*, 321–334.
(21) Baumann, R.; Kumar, A.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.*, in press.
(22) Dubs, A.; Wagner, G.; Wüthrich, K. *Biochim. Biophys. Acta* **1979**, *577*, 177–194.
(23) Wüthrich, K.; Wagner, G. *J. Mol. Biol.* **1979**, *130*, 1–18.
(24) Richarz, R.; Wüthrich, K. *J. Magn. Reson.* **1978**, *30*, 147–150.
(25) Wüthrich, K.; Wagner, G.; Richarz, R.; Perkins, S. J. *Biochemistry* **1978**, *17*, 2253–2263.

They include NOE's between covalently linked protons which are also connected by J-coupling, e.g., along amino acid side chains,[9,10,24,25] between protons in neighboring amino acid residues in the amino acid sequence (e.g., in the β-sheet secondary structure of BPTI[9,10,12,22]), and between different amino acid side chains which are closely spaced in the tertiary protein structure.[9,24,25] It is readily apparent also from the locations of the peaks that there are NOE's between all the different types of protons in the protein, e.g., amide protons with chemical shifts from 8 to 11 ppm,[23] on the one hand, and backbone C^{α} protons from 4 to 6 ppm and aliphatic side chain protons from 0.5 to 4 ppm, on the other hand, or between aromatic protons from 6 to 8 ppm and aliphatic side chain protons, etc. In Figure 2B the previously established[12,22] individual assignments for a small number of NOESY peaks between backbone amide and C^{α} protons have been indicated.

When the spectrum of Figure 2A is compared with previously published NOESY spectra of BPTI, which were recorded with a mixing time of 100 ms,[9,12] it is readily seen that the total number of observable peaks and the relative peak intensities are different throughout the entire spectrum. To investigate the dependence of the spectral features on τ_m in more detail, six NOESY spectra with different mixing times in the range from 20 to 300 ms were recorded. Figures 3 and 4 show the time development of two spectral regions outlined in Figure 2A.

Figure 3 presents three-dimensional views of the spectral region of Figure 2B in the NOESY spectra recorded with different mixing times. The correspondence of the 300-ms trace with Figure 2B can readily be checked. Inspection of the series of six spectra reveals two classes of magnetization transfer exemplified by cross-peaks with different time variations. The first class includes the strong peak between Phe-33 NH and Thr-32 $C^{\alpha}H$ (Figure 2B). The peak intensity increases at short mixing times, goes through a maximum between 100 and 200 ms, and decreases again. These incoherent magnetization transfers are due to dipole–dipole coupling between the protons modulated by the molecular motions, and are the NOE's of interest. The second class is exemplified by the peaks which are shadowed in the spectra recorded with τ_m = 20 and 30 ms, e.g., the peak between NH and $C^{\alpha}H$ of Phe-45. At the short mixing times the intensities of these peaks vary in the form of damped oscillations, which disappear when τ_m gets longer. These oscillations are due to coherent transfer of magnetization by J-coupling between protons, as was recently shown in different systems,[26] and will be further elaborated in the Discussion. Either after the coherent transfer has decayed or even earlier, these peaks reappear with incoherent transfer of magnetization, since the J-coupled protons usually also show NOE's (see the 300-ms spectrum of Figure 3).

Figure 4 shows the τ_m dependence for a cross section through the amide proton resonance of Phe-33 and parallel to ω_1 (Figure 2A). The spectra in Figures 3 and 4 both contain the two cross-peaks from Phe-33 NH to Thr-32 $C^{\alpha}H$ and to Tyr-21 $C^{\alpha}H$. The two classes of magnetization transfer discussed in Figure 3 can also be observed here (a J-coupled cross peak is again shadowed). In addition, the decrease of the diagonal peak with increasing τ_m can be followed. For two methyl resonances, Tyr-32 $C^{\gamma}H_3$ and Ile-19 $C^{\gamma}H_3$, the NOE buildup starts after a lag time of 60 to 100 ms, indicating second-order NOE's through sequential cross-relaxation via protons located between the two protons.[4-7]

Figure 5 shows plots of the peak heights vs. τ_m for peaks in three different cross sections through the spectrum of Figure 2A. Except for the cross-peaks with J-coupling, which have not been included in this presentation, the classes of time behavior discussed above during the description of Figure 3 and 4 can be observed. Distances between the corresponding protons computed from the X-ray structure of BPTI[27] are also indicated in this figure. A qualitative correlation between proton–proton distances and NOE buildup rates is clearly evidenced, with faster rates for shorter

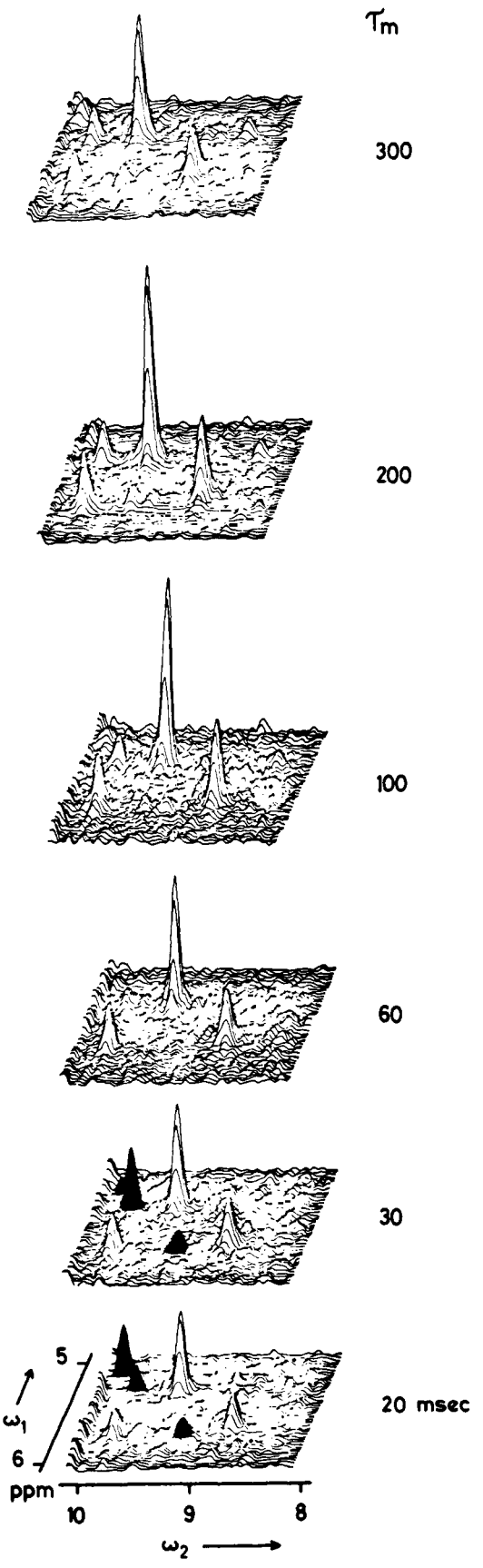

Figure 3. Stacked plots providing a three-dimensional view of the spectral region of Figure 2B in a series of NOESY spectra of BPTI recorded with different mixing times, τ_m, as indicated in the figure. Peaks manifesting NOE connectivities may be identified by comparing the spectrum recorded with τ_m = 300 ms with Figure 2B. The shadowed peaks in the spectra with τ_m = 20 and 30 ms are due to J couplings (see text).

(26) Macura, S.; Huang, Y.; Suter, D.; Ernst, R. R. *J. Am. Chem. Soc.*, in press.

(27) Deisenhofer, J.; Steigemann, W. *Acta Crystallogr., Sect. B* **1975**, *31*, 238–350.

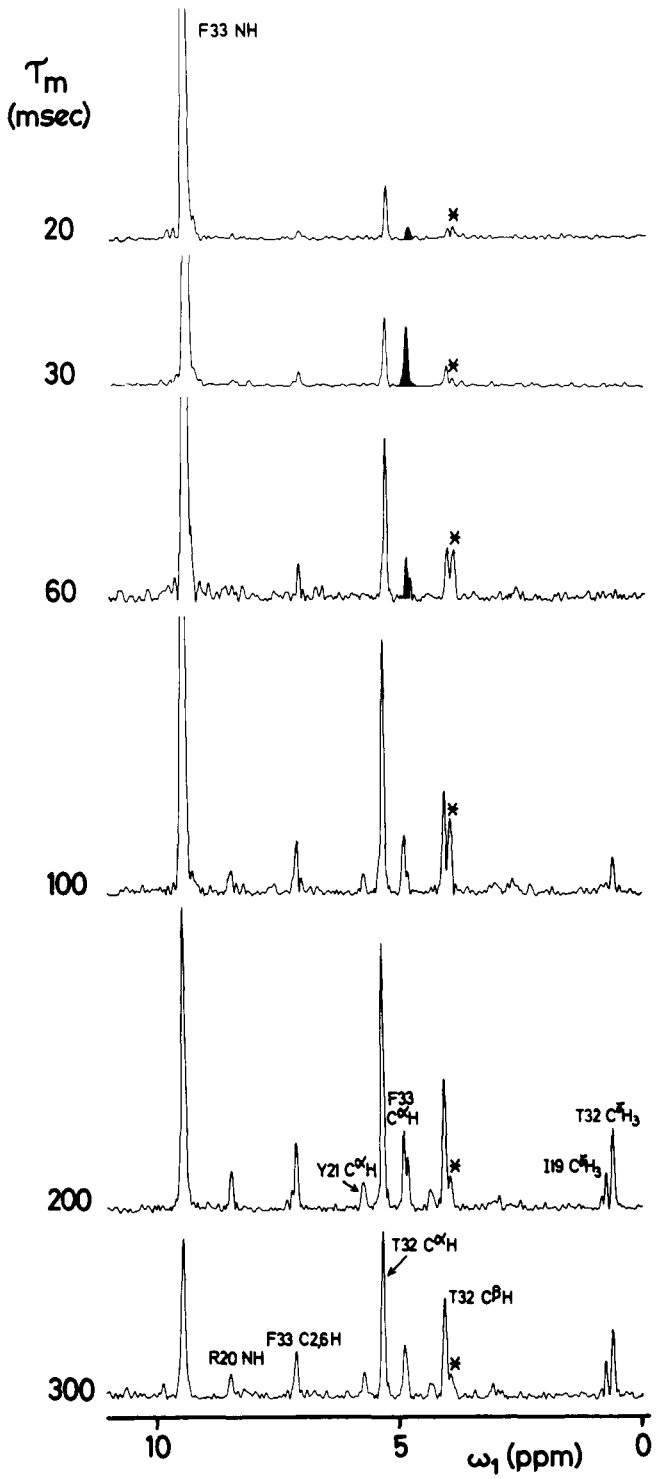

Figure 4. Cross sections parallel to ω_1 and through the diagonal amide proton resonance of Phe-33 at 9.39 ppm (dotted line in Figure 2) from the same series of NOESY spectra of BPTI as shown in Figure 3. Peaks which manifest first-order NOE's are identified in the 300-ms spectrum by the IUPAC-IUB one-letter symbols for amino acids, the position in the amino acid sequence, and the type of protons observed. Additional resonance assignments in the 200-ms spectrum include Tyr-21 $C^{\alpha}H$, Ile-19 $C^{\gamma}H_3$, and Thr-32 $C^{\gamma}H_3$ which show second-order NOE's (see text and Figure 5) and Phe-33 $C^{\alpha}H$, which appears as a strong peak at short mixing times (shadowed in the spectra with τ_m = 20, 30, and 60 ms) due to J coupling with the amide proton of Phe-33 (see text). The peak identified by * corresponds to the tail of a resonance centered in an adjoining cross section.

distances. Furthermore, the two methyl groups which show secondary NOE's are at a distance where first-order NOE's in proteins are hardly effective.[14] Structural analysis of the curves in this figure is discussed in the following section.

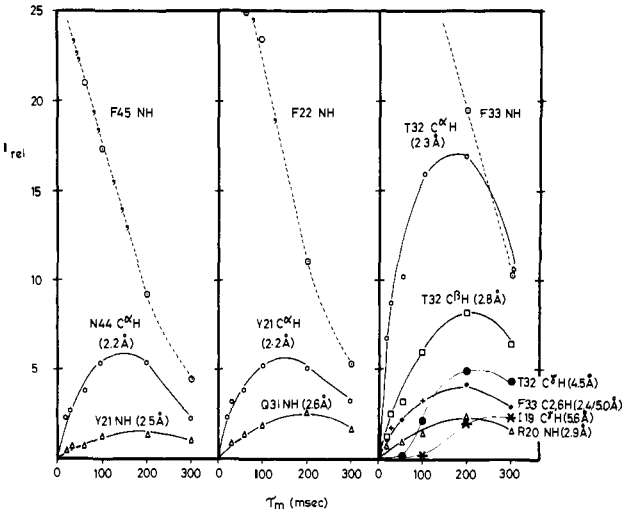

Figure 5. Dependence of the relative peak heights on the mixing time τ_m of cross-peaks in the NOESY spectrum of BPTI (Figures 2–4). The data were measured in cross sections through the diagonal peaks of the amide protons of Phe-45 at 9.98 ppm, Phe-22 at 9.82 ppm, and Phe-33 at 9.39 ppm (Figure 4). The broken lines show the decay of the intensity of the diagonal peaks and are identified by the assignment of these peaks. The time course of the NOE's for protons located near the proton corresponding to the diagonal peak is indicated by the solid curves which connect the experimental points. Resonance assignments and, in parentheses, the proton–proton distances in the X-ray structure[27] are added for each curve, whereby an average proton position was assumed for methyl groups. Two cases of second-order NOE's are clearly evidenced in the diagram on the right, i.e., for Thr-32 $C^{\gamma}H_3$ and Ile-19 $C^{\gamma}H_3$ (see also Figure 4).

Discussion

The nuclear Overhauser effect is one of the few quantities which can be utilized to deduce quantitative information on intramolecular proton–proton distances in molecules in solution. For work with macromolecules, measurements of NOE's must be combined with the high spectral resolution and sensitivity achived only at high frequencies. It is readily apparent from theory[3,4,6] and was borne out by one-dimensional NMR experiments[5-8] that the NOE buildup rates must be studied to obtain the distance information needed for the determination of macromolecular conformations.[12-14] Comparison of the data in Figures 3–5 with the data presented in ref 5 shows that fundamentally the time course of the magnetization caused by cross-relaxation is identical in one-dimensional transient NOE experiments and NOESY. An essential advantage of NOESY, which was already discussed in the introduction to the present paper, is that the problem of limited selectivity of irradiation of individual lines in crowded spectra, which is inherent in one-dimensional techniques, is solved in 2D NMR by the frequency labeling during t_1 and subsequent Fourier analysis. Many aspects of the spectral analysis, however, are closely similar for the two experiments, and some consequences for work with NOESY are discussed in the following.

The τ_m dependence of the peak intensities in NOESY (Figures 3 and 4) is characterized by magnetization buildup in the early phases of the experiment, transition through a maximum, and subsequent decay of the magnetization (Figure 5). The crucial point for the analysis of these curves is that only the initial magnetization buildup can reliably be used for measurements of internuclear distances. For short mixing times τ_m (i.e., in practice $\tau_m \lesssim 100$ ms when working with proteins of molecular weights $\lesssim$20 000), the cross-peak intensities at any given τ_m value are to a good approximation determined by the cross-relaxation rate and by the rate of decay of the magnetization (leakage relaxation) (Figure 5)[16a,c]. From the cross-relaxation rate the proton–proton distance can be calculated. The peak intensity and the τ_m value for the maximum magnetization as well as the time course of the magnetization decay (Figure 5) are further influenced by spin diffusion to additional nuclei.

From Figures 3–5 and the above comments it is clear that variation of the mixing time is indispensable for an unambiguous interpretation and in particular for a quantitative analysis of NOESY spectra. A single 2D spectrum for fixed τ_m value permits at most a qualitative assignment of cross-peaks to pairs of nuclei between which magnetization is transferred during the mixing time. For a more quantitative analysis a set of NOESY spectra for well-selected τ_m values must be performed. At short mixing times, additional cross-peaks occur due to J-coupling.[26] Since the NOE's at short values of τ_m are of most direct interest, it becomes important to distinguish the coherent transfer of magnetization due to J-coupling from the incoherent transfer of magnetization in the NOE cross-peaks.

For a detailed discussion of J cross-peaks, we refer to ref 26. Here we describe only the major features. A number of different pathways exist which can lead to coherent magnetization transfer. They cause characteristic oscillation frequencies by which the J cross-peak intensities vary with the mixing time τ_m. Transfer via zero quantum coherence involves the difference frequency $|\Omega_k - \Omega_l|$, where Ω_k and Ω_l are the Larmor frequencies of the two involved nuclei. Transfer via single quantum coherence, on the other hand, is oscillatory with the characteristic resonance frequencies of the two nuclei, while transfer through double quantum coherence involves the sum frequency $(\Omega_k + \Omega_l)$. All those frequencies are normally quite high and cause the J cross-peak intensities to vary rapidly with the mixing time. J peak intensities are therfore very difficult to predict and suppression or identification of such coherent transfers is imperative for successful, qualitative or quantitative, analysis of NOESY spectra.

Besides the possbilities to identify J cross-peaks by using 2D correlated spectra, such as COSY, SECSY, and FOCSY,[20] two principal possibilities have been proposed to suppress coherent transfer in the NOESY experiment.[26] By suitable phase-shifted pulse sequences, it is possible to selectively compensate J cross-peaks arising from single and double quantum coherence. This possibility utilizes the different behavior of coherence components of different order when a phase shift is introduced.[28] It is, however, not possible to suppress those J cross-peaks which involve zero quantum coherence because these components behave identically with the NOE cross-peaks under phase shifts. A second possibility for the elimination of coherent transfer is by slight random variation of the mixing time τ_m.[26] Random variation of the mixing time within the sequence of t_1 values required for a NOESY spectrum leads to smearing of all J cross-peaks in the ω_1 direction, and random variation of τ_m during accumulation of several transients for a fixed value of t_1 causes the J cross-peaks to be averaged out. The NOE cross-peak intensities, on the other hand, are slowly varying functions of τ_m and are therefore not markedly affected by a slight variation of τ_m.

It is desirable to determine the cross-relaxation rates from the initial buildup rates of the NOE cross-peak intensities since the intensities for longer mixing times τ_m depend also on the leakage mechanisms, like spin diffusion to further nuclei and spin–lattice relaxation, which tend to reduce the transferred magnetization. To obtain the cross-relaxation rates from the initial buildup rates, however, absolute intensity measurements are necessary. Peak intensities are for this purpose unsuitable because they are affected by the peak widths in both frequency dimensions. Broad peaks appear less intense than sharper peaks of the same integrated intensity. This feature is further accentuated by the use of digital resolution enhancement techniques.[29] In the present experiments, the phase shifted sine bell technique[30] has been applied. Therefore, the absolute buildup rates appear to be too low for broad lines, e.g., the amide protons peaks, and quantitative agreement with the proton–proton distances indicated in Figure 5 is rather poor in these cases. For the future, measurement of accurate absolute intensities in NOESY spectra is therefore an important task which may be solved by measuring integrated intensities in pure absorption mode NOESY spectra.

In spite of the respectable time consumption of NOESY experiments for several τ_m values, such studies appear to be extremely promising for investigations of the three-dimensional structure of biopolymers in solution. For the time being there will certainly remain cases where it will be worthwhile to combine NOESY experiments with selective one-dimensional frequency driven NOE experiments.[6] Especially when the resonances are well resolved, the one-dimensional NOE experiments still allow more straightforward intensity measurements and thus more accurate distance estimations and will thus in favorable cases remain useful experiments to complement the two-dimensional techniques.

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(28) Wokaun, A.; Ernst, R. R. *Chem. Phys. Lett.* **1977**, *52*, 407–409.

(29) Ernst, R. R. *Adv. Magn. Reson.* **1966**, *2*, 1.

(30) Wagner, G.; Wüthrich, K.; Tschesche, H. *Eur. J. Biochem.* **1978**, *86*, 67–76.