Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 1-6

A TWO-DIMENSIONAL NUCLEAR OVERHAUSER ENHANCEMENT (2D NOE) EXPERIMENT FOR THE ELUCIDATION OF COMPLETE PROTON-PROTON CROSS-RELAXATION NETWORKS IN BIOLOGICAL MACROMOLECULES

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Received March 19,1980

SUMMARY: The recently developed technique of two-dimensional (2D) cross-relaxation spectroscopy is utilized for systematic measurements of selective nuclear Overhauser enhancements (NOE) in the high resolution ^{1}H nuclear magnetic resonance (NMR) spectra of biological macromolecules in solution. Compared to conventional one-dimensional NOE studies, the 2D NOE experiment has the principal advantage that it avoids detrimental effects arising from the limited selectivity of preirradiation in crowded spectral regions. Furthermore, it yields with a single instrument setting a complete network of NOE's between all the protons in the macromolecule. The resulting information on intramolecular proton-proton distances provides a new avenue for studies of the spatial structures of biopolymers.

Recent technological developments resulted in important improvements of the spectral resolution (1,2) and the possibilities to delineate J-coupling connectivities (3-5) in the ^{1}H nuclear magnetic resonance (NMR) spectra of macromolecules. At this stage the utility of high resolution NMR for studies of biopolymer conformations depends further critically on procedures to correlate NMR parameters with spatial macromolecular structures (6). Selective ^{1}H-^{1}H nuclear Overhauser effects (NOE) manifest the distances between different fragments of a polymer chain, which can be directly related to the molecular conformation (7-9). This paper describes the first use of two-dimensional (2D) cross-relaxation spectroscopy (10-12) for measurements of intramolecular NOE's and presents data obtained with a small globular protein, the basic pancreatic trypsin inhibitor (BPTI).

The NOE is the fractional change in intensity of one NMR line when another resonance is perturbed and has long been a valuable tool for structural studies of small molecules (13). In macromolecules at high magnetic fields, however, spin diffusion can become quite efficient (7,14,15), causing the conventional steady-state NOE's (13) to be less specific and hence less useful. Theory

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0006-291X/80/130001-06$01.00/0

shows that, in contrast, the initial build-up rates of NOE's are simply related to the inverse sixth power of the distance between the observed and the presaturated proton (7-9,13-16). One-dimensional experiments for measurements of NOE build-up rates have been developed (7,9). Their practical use is limited, however, since they are rather time-consuming and because of the poor selectivity for preirradiation of individual resonance lines in crowded regions of the ^{1}H NMR spectra. These practical difficulties can be largely overcome with the use of the 2D NOE experiment described in the following.

PRINCIPLES OF THE 2D NOE TECHNIQUE: The 2D NOE experiment uses a recently developed 2D NMR method for investigations of cross-relaxation and chemical exchange processes (10,11). As shown in the scheme of Fig. 1 the experiment consists of a sequence of three non-selective 90° pulses. During the evolution time between the first and the second pulse, t_1, the various magnetization components are frequency-labelled. During the mixing period between the second and the third pulse, τ_m, cross-relaxation leads to exchange of magnetization between nearby protons through mutual dipolar interactions. The interval τ_m is kept fixed and the signal recorded immediately after the third pulse as a function of t_2. In accordance with the general 2D spectroscopy principle (17) the experiment is repeated for a set of equidistant t_1 values. A two-dimensional Fourier transformation of the data matrix $s(t_1,t_2)$ then produces the desired frequency domain spectrum.

The general features of a 2D NOE spectrum are outlined in the lower part of Fig. 1. Magnetization components which do not exchange with other components during the mixing time τ_m have the same frequencies during t_1 and t_2. Hence the corresponding peaks in the ω_1-ω_2 spectrum lie on the diagonal which disects the two frequency axes. Exchange of magnetization between two components due to dipolar coupling during the mixing period is manifested by cross-peaks between the coupled components. In the scheme of Fig. 1 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. The fundamental aspects of this experiment have recently been worked out by Macura and Ernst (12).

EXPERIMENTAL: Application of the 2D NOE technique for studies of biological macromolecules requires the handling of particularly large data matrices. For this a slightly modified version of the software previously developed for recording 2D J-resolved ^{1}H NMR spectra of macromolecules (1,2) was used. 2D NOE spectra were recorded on a Bruker HX-360 spectrometer equipped with an Aspect 2000 data system. The basic pancreatic trypsin inhibitor (BPTI, Trasylol®) was obtained from the Farbenfabriken Bayer AG. Further experimental details are given in the figure caption 2.

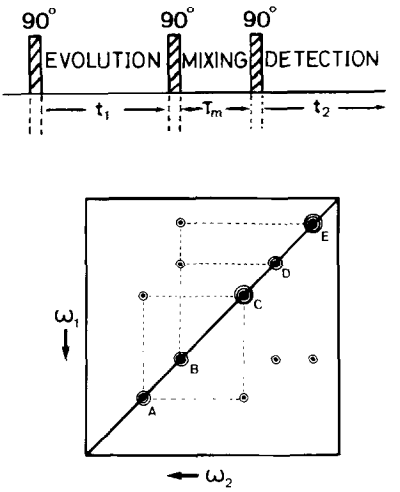

Fig. 1 The top trace shows a scheme for the 2D NOE experiment, which consists of a sequence of three 90° pulses. The pulses are separated by the evolution period, t_1, and the mixing period, τ_m, respectively. Immediately after the third pulse, the signal $s(t_1,t_2)$ is recorded. The bottom trace shows a contour plot of a schematic 2D NOE 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.

RESULTS: A proton 2D NOE spectrum of the globular protein BPTI is shown in Fig. 2. Experimental details are given in the figure caption. The spectral range in each of the two dimensions, ω_1 and ω_2, extends from 1 to 10 ppm, so that with the exception of the lowest field amide proton line at 10.55 ppm all the resonances of BPTI are contained in Fig. 2. The distribution of signal intensity of the chemical shift axis seen previously in conventional one-dimensional ^{1}H NMR spectra of BPTI (6,19) is faithfully manifested on the diagonal from the lower left to the upper right corner, as one would have expected from the scheme in Fig. 1. Bands of intense signals extend from the water line at 4.85 ppm parallel to both the ω_1 and ω_2 axes. This is mainly due to the dispersion mode signal contained in the absolute value plot shown here. The informative feature of the spectrum are the cross-peaks seen throughout the spectral plane. On the basis of previously established resonance assignments in BPTI (19) and from comparison with earlier one-dimensional NOE studies (20-22) many of these cross-peaks have been identified as selective NOE's between individual groups of protons which manifest intramolecular proton-proton distances.

To illustrate how a 2D NOE spectrum is analyzed, some previously established (20-22) NOE connectivities are indicated by the broken lines in Fig. 2. The backbone amide proton of Glu 31 is connected with the α-proton of Cys 30. The

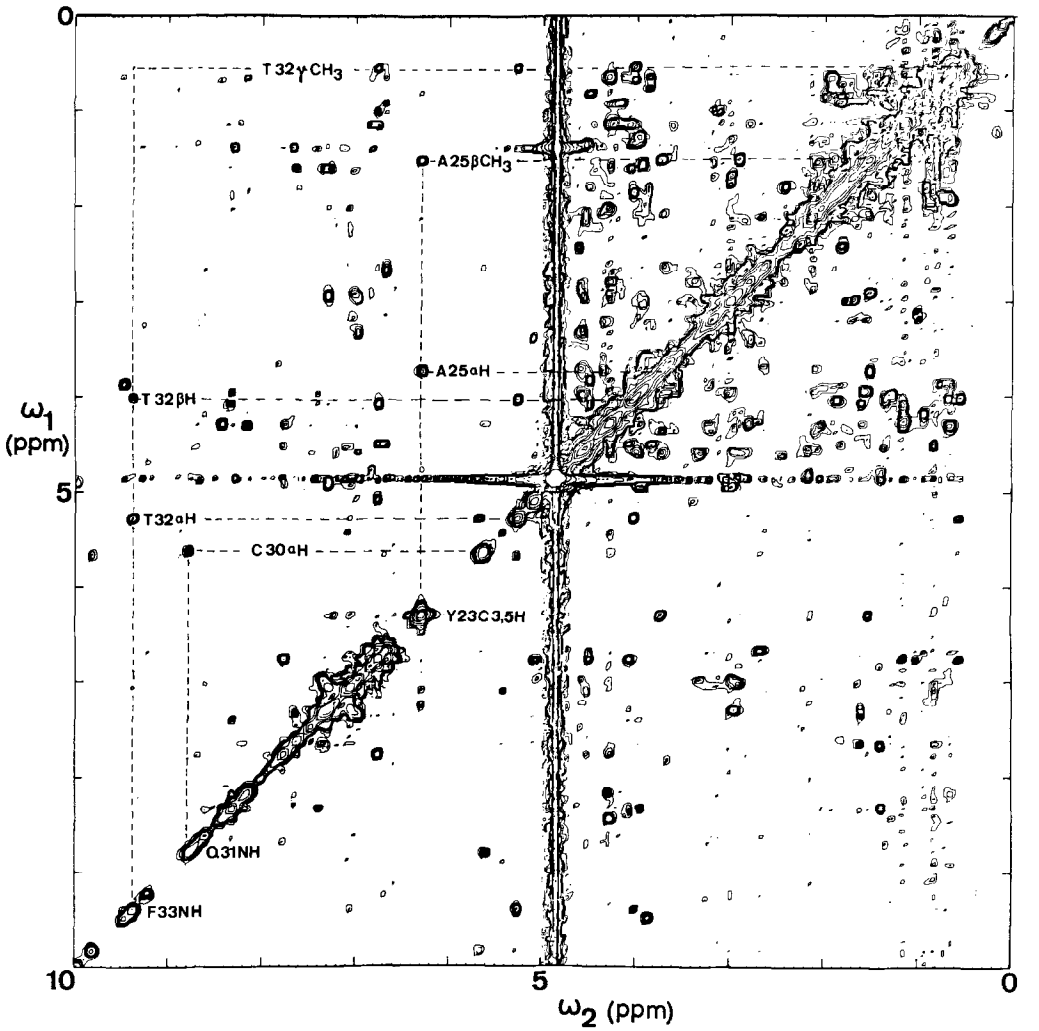

Fig. 2 Contour plot of a proton 2D NOE spectrum at 360 MHz of the basic pancreatic trypsin inhibitor. The protein concentration was 0.02 M, solvent ^2H_2O, p^{2}H=3.8, T=18°C. The spectral width was 4000 Hz. 512 data points were used in each dimension. 56 transients were accumulated for each value of t_1. The mixing time τ_m was 100 msec. The total accumulation time was 18 hours. The absolute value spectrum obtained after digital filtering in both dimensions with a shifted sine bell (18) is shown. Cross-relaxation connectivities for selected amino acid residues are indicated by the broken lines (see text). Connected peaks are identified by the one-letter symbol for amino acids (A=alanine, T=threonine, C=cysteine, Q=glutamine, F=phenylalanine, Y=tyrosine), the position in the amino acid sequence and the type of protons observed.

connectivity is manifested by a second cross-peak in a symmetrical location with respect to the diagonal (see also Fig. 1). NOE's between the 3,5 ring protons of Tyr 23 and the α- and methyl protons of Ala 25 (21) give rise to strong cross-peaks. The NOE's between the amide proton of Phe 33 and Thr 32

are manifested by strong cross-peaks for the α- and β-protons of Thr 32, and a weak peak for the methyl protons. All the NOE's previously observed by one-dimensional experiments have been seen in the 2D NOE spectra and at the present early stage of the spectral analysis numerous new connectivities have already been established. These will be further discussed elsewhere.

Fundamentally, the 2D NOE spectrum manifests for all possible combinations of protons in the macromolecular structure the phenomena seen in a conventional transient NOE experiment (7) for selected individual protons. After a mixing time, τ_m, of 100 ms the strongest cross-peaks are due to direct cross-relaxation between protons located at distances smaller than ca. 3.0 Å (22). With a shorter mixing time τ_m one would further emphasize the very short proton-proton distances (7,9,22). With the use of longer mixing times the intensities of the cross-peaks due to direct dipolar coupling between nearby protons would decrease on account of new peaks arising from spin diffusion. Hence, by recording 2D NOE spectra with a short mixing time we obtain a map of all the short proton-proton distances in a macromolecular structure. These can readily be correlated with the spatial molecular structure. Further studies are needed to explore how the information on indirect cross-relaxation pathways, "spin diffusion", obtained with longer mixing times can be employed for studies of static and/or dynamic aspects of protein conformations.

CONCLUSIONS: Compared to conventional one-dimensional experiments for measurements of selective proton-proton NOE's in macromolecules the 2D NOE experiment used in this paper has the following principal advantages: (i) With a single instrument setting it provides a complete set of NOE's between all closely spaced groups of protons in the macromolecular structure. (ii) It avoids adverse effects arising due to non-selective preirradiation of nearby resonances in crowded spectral regions. (iii) A complete set of NOE's is obtained with a significant saving in time. Since the NOE's measured with a suitably chosen mixing time τ_m (Fig. 1) can be directly related with interatomic distances in spatial molecular structures (7-9,22) the easy availability of NOE data from 2D NOE experiments promises to open new avenues for studies of biopolymer conformations.

ACKNOWLEDGEMENTS: This project is financed by a research grant of the ETH, Zürich. We would like to thank Drs. K. Nagayama and E. Bartholdi for help with the software modifications and Drs. L.R. Brown and G. Wagner for helpful discussions on the interpretation of the spectral data.

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