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A NOVEL APPLICATION OF NUCLEAR OVERHAUSER ENHANCEMENT (NOE) IN PROTEINS: ANALYSIS OF CORRELATED EVENTS IN THE EXCHANGE OF INTERNAL LABILE PROTONS

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SUMMARY: A quantitative evaluation of nuclear Overhauser enhancements between slowly exchanging labile protons in a globular protein can be used to decide between correlated and uncorrelated exchange processes. The method appears to be useful for distinction of EX_1 and EX_2 mechanisms. Demonstrations are given with experiments on the basic pancreatic trypsin inhibitor.

In globular proteins, nuclear Overhauser enhancement (NOE) measurements have been used to detect close spatial proximity of different atoms, to estimate intramolecular distances and to study the phenomenon of spin diffusion (1-12). A further application of NOE which is suggested in this paper appears to be very useful for distinction of different kinetic mechanisms in the process of amide proton exchange.

THEORY: We consider two protons A and B which are close neighbours in the 3-dimensional structure of a globular protein. Fig. 1 shows a typical situation for two amide protons of an antiparallel β-pleated sheet. The observed magnitude of the NOE between the two protons is determined by their distance, the overall and internal mobility of the protein, and the instrumental conditions used. In general the position of other nearby nuclei will influence the NOE, however, by proper choice of instrumental conditions this contribution to the observed NOE can be strongly reduced (10,11).

In this paper NOE's are measured by difference spectroscopy using the following pulse sequence:

$$(-\tau_1(\omega_A)\text{-observation pulse-}\tau_2\text{-}\tau_1(\omega_{\text{off-res}})\text{-observation pulse-}\tau_2\text{-})_n.$$

A selective low power radiofrequency field is applied to resonance A for a period of time τ_1, which is followed immediately by the observation pulse. After a waiting time τ_2, a reference spectrum without NOE is

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recorded. The free-induction decays with and without NOE are stored in different parts of the memory, where they are accumulated alternately. The final free-induction decays are subtracted and the difference is Fourier transformed to obtain the NOE difference spectrum (10). In the difference spectrum the intensity of the irradiated resonance of proton H_A is denoted by I_A, and the resonance intensity of the neighbouring proton H_B, which is due to NOE, is denoted by I_B. The ratio of I_B and I_A is denoted by $\tilde{\eta}_{AB}$ as magnetization transfer:

$$\tilde{\eta}_{AB} = \frac{I_B}{I_A}$$

If both signals have equal intensity in the reference spectrum $\tilde{\eta}_{AB}$ is identical with the NOE.

If we work in ^2H_2O solution, amide protons may exchange against deuterium with time, and the probability of sites A and B being protonated is $p_A(t)$ and $p_B(t)$, respectively ($p_A(t)$, $p_B(t) \leq 1$). $p_A(t)$ and $p_B(t)$ can be determined from the intensities of the signals in the reference spectrum. The NOE difference spectrum yields information about $p_{AB}(t)$, the probability that both sites are protonated:

$$I_B(t) = p_{AB}(t) \cdot I_B(0)$$

and

$$\tilde{\eta}_{AB}(t) = \frac{I_B(t)}{I_A(t)} = \frac{p_{AB}(t)}{p_A(t)} \cdot \tilde{\eta}_{AB}(0)$$

In this context $p_{AB}(t)/p_A(t)$ is the quantity of interest, $\tilde{\eta}_{AB}(0)$ is a calibration constant obtained from a reference experiment with a fresh sample where $p_A(t) = p_B(t) = 1$. After a certain time t_1 sites A and B will be partially deuterated and $p_A(t_1)$, $p_B(t_1) < 1$. The NOE measured in this aged sample depends on the mechanism which was responsible for exchange:

a) <u>uncorrelated exchange:</u> If the exchange of H_A and H_B is uncorrelated,

$$p_{AB}(t_1) = p_A(t_1) p_B(t_1)$$

and

$$\tilde{\eta}_{AB}(t_1) = p_B(t_1) \tilde{\eta}_{AB}(0)$$

Thus the original magnetization transfer is reduced by the factor $p_B(t_1)$.

b) correlated exchange: If H_A exchanges only together with H_B and vice versa, then either both sites are protonated or both sites are deuterated. Then

$$p_A(t_1) = p_B(t_1) = p_{AB}(t_1)$$

and

$$\tilde{\eta}_{AB}(t_1) = \tilde{\eta}_{AB}(0)$$

Thus the magnetization transfer remains unchanged after t_1.

To minimize spin diffusion effects the experimental conditions used should generally be those of a truncated Overhauser enhancement experiment (TOE) as described in (10). This is essential since almost always other labile protons are in close proximity to H_A and H_B and the pathways of spin diffusion might be changed by partial deuteration of these other sites.

NH EXCHANGE MECHANISMS: The exchange of internal amide protons can be described by the following equation (13-16).

$$N(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow[^2H_2O]{k_3} O(^2H) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N(^2H)$$

The closed state N(H) is in equilibrium with open states O(H). The exchange is possible only in the open states O(H) and leads, in the presence of ^2H_2O, to deuteration of the peptide site. The experimentally observable overall exchange rate k_m is approximately:

$$k_m = \frac{k_1 k_3}{k_2 + k_3}$$

There are two limiting kinetic situations:

a) EX_1 process

If $k_3 >> k_2$ each opening of the closed state N leads to an isotope exchange and we have:

$$k_m = k_1.$$

If two amide protons are adjacent in the 3-dimensional structure of the protein and opening exposes both labile protons simultaneously, both protons should exchange in a correlated way.

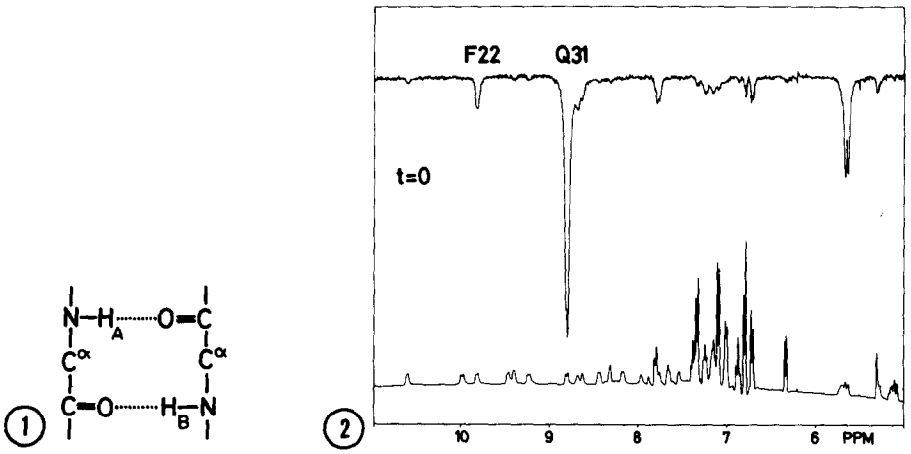

Fig. 1 Schematic representation of two adjacent hydrogen bonds connecting opposite strands of an antiparallel β-sheet. The two amide protons H_A and H_B are separated by approximately 2.6 Å

Fig. 2 360 MHz NOE difference spectrum (4000 scans) of a 20 mM solution of the basic pancreatic trypsin inhibitor in ^2H_2O at p^{2}H 4.6, 24° C. Lower trace: reference spectrum; upper trace: NOE difference spectrum. τ_1 = 500 msec, τ_2 = 1 sec.

b) EX_2 process

If $k_3 << k_2$ only a small portion of all openings leads finally to the exchange of internal protons and

$$k_m = \frac{k_1}{k_2} \cdot k_3 .$$

Since in this case the exchange is not dominated by the opening rate k_1, which is likely to be a common parameter for two adjacent protons no correlation is imposed on the exchanging protons.

MATERIALS AND METHODS: The basic pancreatic trypsin inhibitor BPTI (Trasylol ^{R} Bayer Leverkusen) was obtained as a gift from Farbenfabriken Bayer AG. 20 mM solutions of the protein in ^2H_2O were used for the NMR measurements. ^1H NMR spectra were recorded on a Bruker HX 360 instrument in the Fourier mode. Typically 4000 scans were accumulated for one NOE difference spectrum. Chemical shifts are quoted relative to internal sodium-2,2,3,3-tetradeutero-3-trimetylsilyl-propionate.

RESULTS: The main structural feature of the basic pancreatic trypsin inhibitor is a twisted antiparallel β-sheet (17). Most of the NMR signals of the hydrogen bonded amide protons of this β-sheet were assigned previously (11,18). Fig. 2 shows a NOE experiment with a fresh sample at 24° C and p^{2}H 4.6. Under these conditions all internal amide protons ex-

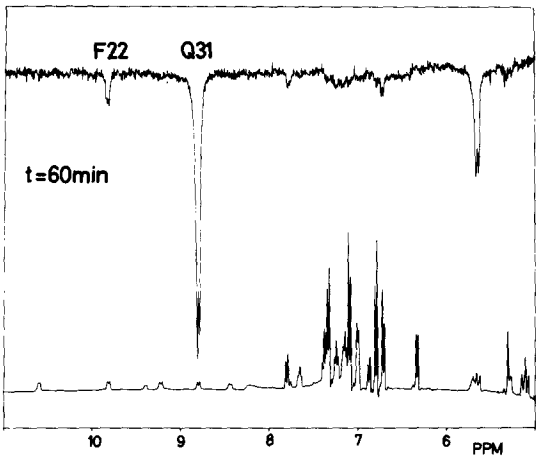

Fig. 3 Same as Fig. 2 but prior to the experiment the sample was kept at 60°, p^2H 8.0 for one hour to partially exchange the amide protons.

change extremely slowly (15). Only the low field part of the spectrum is shown (5-11 ppm). The lower trace is the reference spectrum, the upper trace the NOE difference spectrum. The NH resonance of Gln 31 at 8.78 ppm which corresponds to H_A in Fig. 1 was saturated for 500 msec prior to each scan. The strong NOE of 40 % at 5.63 ppm corresponds to the α-proton of Cys 30, which is the closest proton to the NH of Gln 31 (11,17). Among the amide protons, the resonance of Phe 22 at 9.79 ppm shows the strongest NOE (13 %) since Phe 22 lies face to face with Gln 31 in the β-sheet (H_B in Fig. 1).

In a second experiment, a 20 mM solution of the protein was kept for 1 hour at 60° at p^2H 8.0. After this time the peptide sites of Phe 22 and Gln 31 were approximately half deuterated ($p(t_1) \approx 0.5$). To stop further exchange the temperature was lowered to 24° and the p^2H was changed to 4.6. A NOE experiment corresponding to that of Fig. 2 was performed on this sample (Fig. 3). The non labile α-proton of Cys 30 shows an almost identical NOE (45 %) confirming that the instrumental conditions of the first experiment were reproduced. A quantitative measurement of the resonance intensities of the amide proton lines shows that almost identical NOE (14 %) is again observed at the NH of Phe 22. Thus, at p^2H 8.0 and 60°, the internal amide protons of Phe 22 and Gln 31 exchange in a fully correlated process, indicating EX_1 exchange.

DISCUSSION: Measurements of exchange rates of internal amide protons are a common approach for studying the dynamics of biological macromolecules. For meaningful interpretation of such data, it is essential to know whether the exchange follows an EX_1 or an EX_2 mechanism. In the EX_2 process, k_m is proportional to k_3. Thus k_m should be proportional to the concentration of $^2H_3O^+$ or OH^- in the acid or base catalyzed regime, respectively, modulated only by the p^2H dependence of the equilibrium constant k_1/k_2. For the EX_1 process on the other hand, the only p^2H dependence of k_m is that of the opening rate constant k_1 which is not known a priori. Thus distinction between EX_1 and EX_2 processes has so far been based on the p^2H dependence of the exchange rate k_m. A linear increase with slope 1 for log k_m vs p^2H in the base catalyzed regime was taken as evidence for an EX_2 process. This criterium fails if, by chance, k_1 has a similar p^2H dependence as k_3. NOE studies as described in this paper appear to be an independent additional criterium to identify EX_1 exchange. A systematic analysis of the exchange of labile protons in the basic pancreatic trypsin inhibitor by this method is in progress.

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