Biochemical Pharmacology, Vol. 40, No. 1, pp. 115–119, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

Determination of ligand conformation in macromolecular complexes using the transferred nuclear Overhauser effect

The transferred nuclear Overhauser effect (TRNOE) [1, 2] represents an extension of NOE measurements on biological macromolecules, such as small proteins and nucleic acid fragments, to exchanging systems, in particular protein–ligand complexes. The basis of the TRNOE involves the transfer of magnetic information concerning cross-relaxation between two bound ligand protons from the bound state to the free state via chemical exchange. In this manner negative NOEs on the easily detectable free or averaged ligand resonances can be observed following the pertubation of the magnetization of other ligand resonances (free, bound or averaged), thus providing information on the proximity in space of two bound ligand protons. In the presence of a macromolecule for which $\omega\tau_c \gg 1$ (the spin diffusion limit), a negative TRNOE will be observed on either the free or averaged ligand resonance i following perturbation (e.g. selective irradiation in a one-dimensional experiment) of the free, bound or averaged resonance of ligand proton j whose size is proportional to the cross-relaxation rate between the two protons in the bound state if the following conditions are met:

$$k \geq 10\rho_{iF}$$

and

$$|(1 - a)\sigma_{ij}^{BB}| \gg |a\sigma_{ij}^{FF}|$$

where k is the chemical exchange rate between the free and bound ligand, ρ_{iF} the spin-lattice relaxation rate of proton i in the free state, a the mole fraction of the free ligand, and σ_{ij}^{FF} and σ_{ij}^{BB} the cross-relaxation rates between protons i and j in the free and bound states respectively [1]. Figure 1 schematically illustrates the principle of the TRNOE and presents a simple example.

In practical cases involving large proteins, steady-state TRNOE measurements would not be selective due to highly effective indirect cross-relaxation between many protons, a process known as spin-diffusion. One simple and practical approach to circumvent associated problems consists of carrying out systematic pre-steady-state TRNOE measurements throughout the whole spectral region, yielding an "action spectrum" in which apparent selectivity is maintained. Examples of such "action spectra" are presented in Fig. 2. Although very effective, this approach, however, is limited since one can only determine the distance ratio from two protons to a third proton because cross-relaxation rates between pairs of bound ligand protons cannot be obtained from such measurements at a single irradiation time.

This limitation can be overcome by measuring the initial slope of the buildup curves of the time-dependent TRNOE from which cross-relaxation rates between pairs of bound ligand protons can be obtained directly since

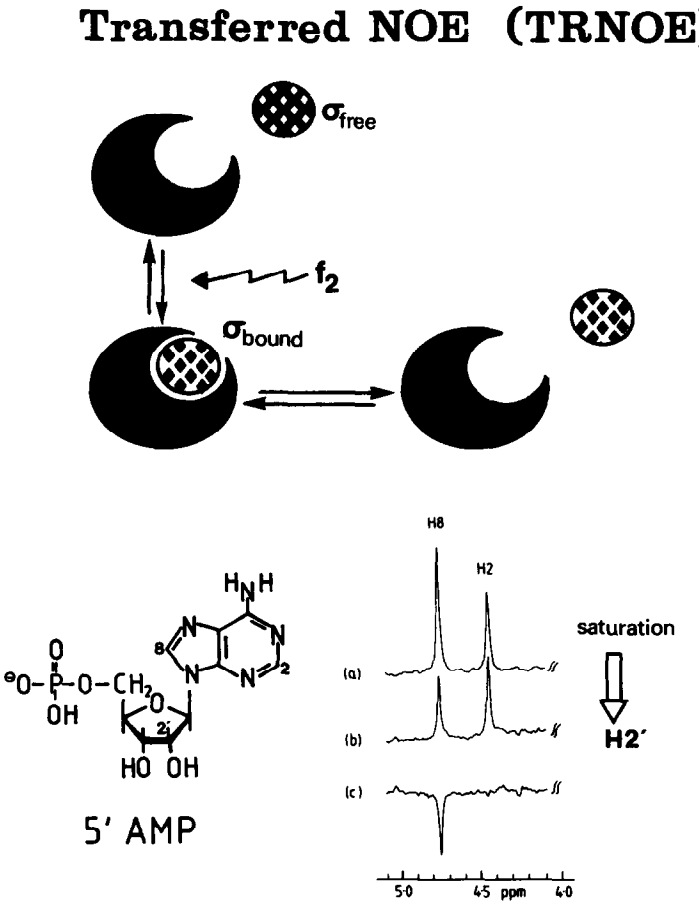

Fig. 1. Schematic representation of the TRNOE. The spectra show the aromatic region of the 270 MHz ^{1}H-NMR spectrum of 3.33 mM 5'-AMP in the presence of 0.1 mM yeast ADH: (a) control irradiation; (b) irradiation at the observed H2' sugar resonance; and (c) spectrum (b) minus spectrum (a).

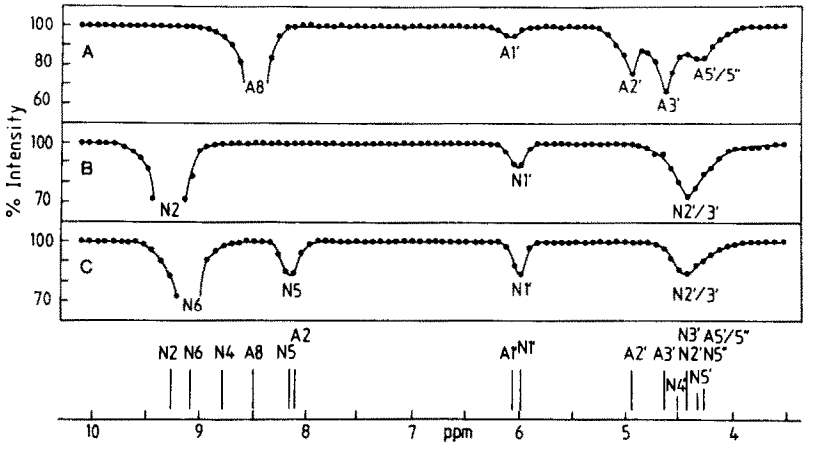

Fig. 2. TRNOE "action spectra" monitoring the intensity of the averaged H_A8 (A), H_N2 (B), and H_N6 (C) resonances of $NADP^+$ in the complex with yeast glucose-6-phosphate dehydrogenase.

$$\left.\frac{dM_i}{dt}\right|_{t=0} = -[(1-a)\sigma_{ij}^{BB} + a\sigma_{ij}^{FF}]$$

providing $\sigma_{ij}^{BB} \geq \sigma_{ik}^{BB}$, σ_{ls}^{BB} or $\sigma_{ij}^{BB} \geq \sigma_{jk}^{BB}$, σ_{js}^{BB}. The presence of indirect cross-relaxation in the absence of direct cross-relaxation becomes easily apparent by a pronounced lag phase in the time dependence of the TRNOE, thus precluding the measurement of an initial slope and providing no information about the respective distances [2].

Numerous examples of the application of the TRNOE have appeared in the literature [3–24], mostly dealing with the conformation of small ligands, such as nucleotides, bound to proteins. Two studies on nucleic acid complexes [21, 22] and some on peptide–protein complexes [23, 24] have been reported. The TRNOE technique is at its most powerful when combined with structural data from X-ray crystallography and, as an example for this strategy, a study on ATP and GTP bound to carp haemoglobin [8] is summarized briefly. This particular study was undertaken to find out why GTP is a more potent allosteric effector than ATP in teleost fish haemoglobin. Although there is a detailed X-ray structure of human deoxyhaemoglobin available [25], no crystal structure of a fish haemoglobin has been solved yet, preventing a structural analysis of the bound nucleotide in the active site. We therefore determined the conformation of ATP and GTP bound to carp deoxyhaemoglobin by the TRNOE method, and the time dependence of the TRNOE for several protons on ATP is presented in Fig. 3. It was found that both ATP and GTP in their bound form have a high *anti* conformation about the glycosidic bond, the sugar ring is 3'-endo and the P-O5'-C5'-C4' torsion angle lies in the *trans* domain. Models for the nucleoside triphosphates were fitted using interactive graphics and energy refinement into a model of human deoxyhaemoglobin in which the side chains in the NA, EF and H segments were replaced by those of the carp sequence. An excellent fit of both nucleotides into the

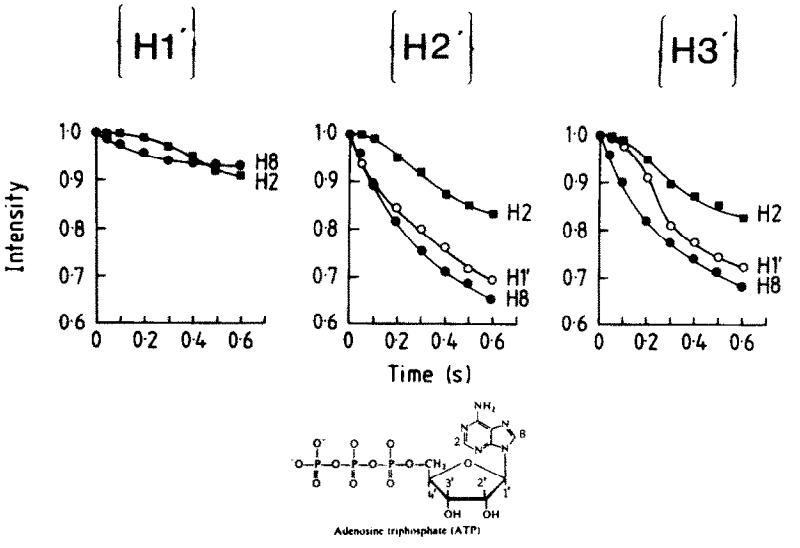

Fig. 3. Time dependence of TRNOEs observed on the averaged H8, H2 and H1' resonances of ATP following irradiation of the averaged H1', H2' and H3' resonances of ATP in the presence of carp deoxyhaemoglobin.

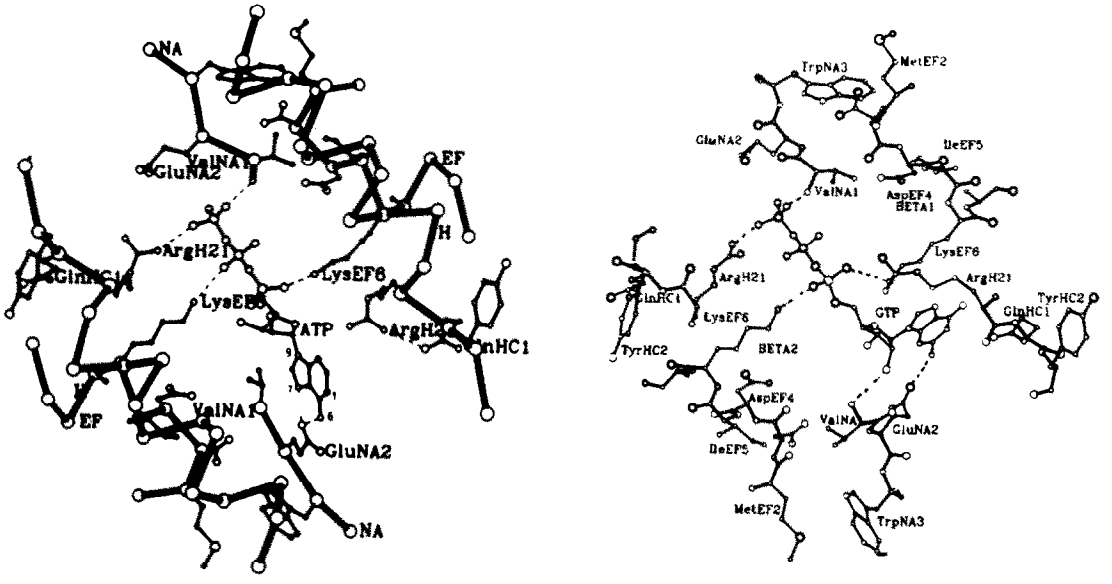

Fig. 4. Proposed binding of ATP and GTP to carp deoxyhaemoglobin derived from TRNOE measurements and molecular modelling.

active site was found with the 6-amino group of ATP being within hydrogen bonding distance of Glu NAβ_1, and GTP, after rotation about 180° along its long axis, being able to donate a hydrogen bond from the 2-amino group to the same Glu side chain. In addition, Val Na1β_1 can donate a hydrogen bond to the O2′ of the ribose ring of GTP. Thus, the presence of an additional hydrogen bond in the case of GTP, as compared to ATP, provides an explanation for the observation that GTP is a better allosteric effector than ATP. Figure 4 shows the models for ATP and GTP bound in the active site of carp deoxyhaemoglobin.

As a second example, the structure of the tetrapeptide acetyl-Pro-Ala-Pro-Tyr-NH_2 bound to porcine pancreatic elastase [23] is presented. In this case 23 approximate distance restraints between pairs of bound ligand protons were derived from two-dimensional TRNOE measurements. 2D TRNOE spectra of the tetrapeptide–elastase complex with a ligand to protein ratio of 11 to 1 are presented in Fig. 5. The structure of the bound peptide was refined by restrained molecular dynamics [26] from

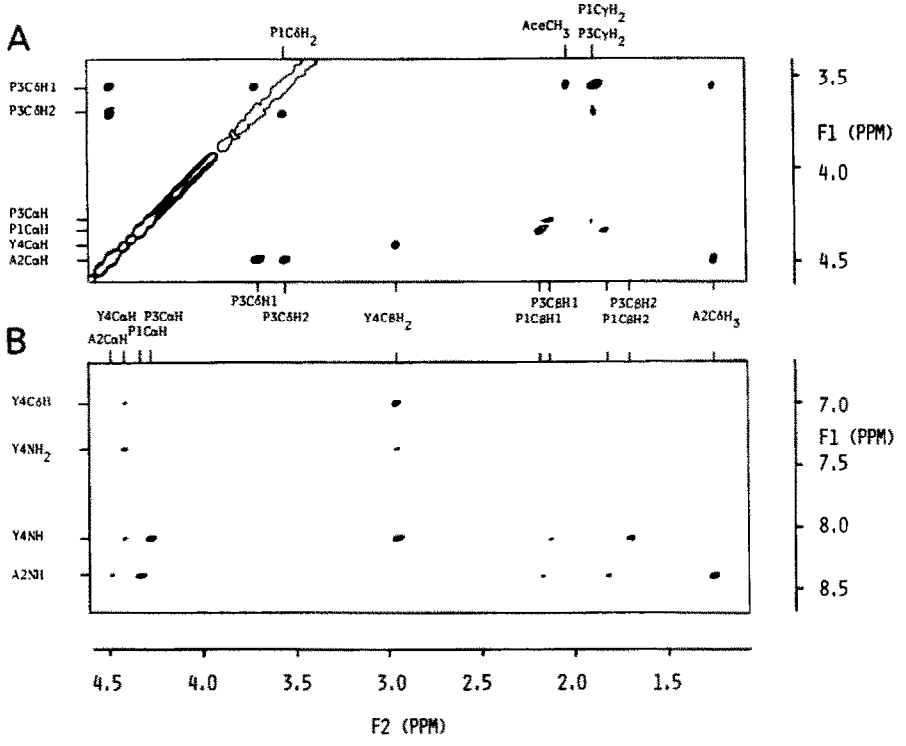

Fig. 5. Two-dimensional TRNOE spectra of acetyl-Pro-Ala-Pro-Tyr in the complex with porcine pancreatic elastase: (A) 99.96% ^2H_2O buffer, and (B) 90% H_2O/10% ^2H_2O buffer.

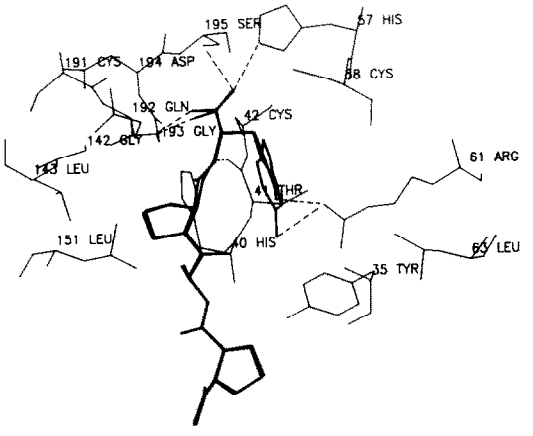

Fig. 6. Proposed binding of acetyl-Pro-Ala-Pro-Tyr in the S′ leaving group location of elastase derived from TRNOE measurements and molecular modelling.

different starting structures, and the converged final structure was modelled into the active site of elastase by interactive molecular graphics. The determination of the anchor point of the bound inhibitor was aided by a simultaneous crystallographic study which, despite the fact that only electron density for a Pro-X dipeptide fragment was visible, enabled both the approximate position and orientation of binding to be determined. It was discovered that the peptide inhibitor was bound in the S′ binding site in the reverse orientation found in other serine protease inhibitor complexes, and a view of the peptide in the active site is shown in Fig. 6. This study demonstrates most strikingly that data from NMR studies (namely the conformation of the bound ligand) and X-ray crystallography (namely the approximate location of the ligand and the structure of the enzyme alone) in combination with molecular modelling may be extremely useful for the development of drug design methodologies.

In conclusion, the TRNOE method is a powerful method for the determination of ligand conformations in systems where chemical exchange occurs between the bound and free ligand. It works most effectively and easily for weakly binding ligands with a resonably fast off-rate ($>10^3\,sec^{-1}$). Advantages of the TRNOE are that the measurements can be carried out on the easily detectable and assignable free/averaged ligand signals, that the sensitivity of the experiment increases with the molecular weight of the complex studied (since $\sigma_{ij} \propto \tau_c$), and that a large excess of ligand can be used for large complexes.

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Biochemical Pharmacology, Vol. 40, No. 1, pp. 119–123, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Probing the role of proline as a recognition element in peptide antigens

The role of the imino acid *S*-proline in controlling local conformation in oligopeptides is a topic of much pharmacological interest. Recent work on the design and construction of synthetic vaccines, and of antagonists to peptides such as bradykinin, has revealed its importance in controlling molecular structure responsible for observed biological activity. Additional impetus to research on this structural problem has been provided by the startling discovery that cyclophilin, a protein that binds the immunosuppressive drug cyclosporin A, can catalyse the slow isomerisation of X-Pro amide bonds in oligopeptides [1, 2]. We have attempted to probe the molecular mechanism by which proline exerts its conformational influence using a series of non-natural amino acid replacements in studies on the recognition of small peptides by monoclonal antibodies. Our interest in this topic arose from research aimed at detailing the molecular interactions responsible for the recognition of an immunogenic nonapeptide, H_2N-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-OH, **1**, (Fig. 1). This sequence occurs in the HA1 subunit of influenza virus hemagglutinin and has been shown to be highly immunogenic in raising antipeptide antibodies able to bind to the intact protein [3]. Two monoclonal antibodies (DB19/1 and DB19/25) were raised which tightly bound the nonapeptide **2**, which is related to **1** by acetylation of the N-terminal amino group. Significantly, it was determined, using standard amino acid replacement methods, that the recognition of **2** by the antibodies was dependent mainly upon the Tyr-Pro-Tyr-Asp segment of the peptide.* As NMR evidence had been reported indicating that, in aqueous solution, **1** exists in a highly populated conformation in which the residues Tyr-Pro-Tyr-Asp occupied a defined secondary structure (type II β-turn) [4], we decided to explore this segment of the peptide in more detail. In particular, the correlation between recognition and conformational constraints introduced into the oligopeptide by the *S*-proline residue in this region was investigated using a quantitative binding assay of the recognition of nonapeptide analogues **3** and **4** by DB19/1 and DB19/25. In these peptides, *S*-proline was replaced by the non-natural residues *S*-2-methylproline (2-MePro) and *S*-*N*-methylalanine (NMeAla) respectively. In both cases, the introduction of these analogue amino acids gave oligopeptides which were similar in their charge and steric properties with respect to **2**. However, we anticipated that the conformational dynamics of **3** and **4** would be altered significantly compared to that of **2** in the important tetrapeptide segment.

Molecular modelling was used to determine the extent to which the conformational minima of the peptide backbones of **2** and **3** were a subset of those available to **4**. Our results suggested that there was no strain energy penalty incurred upon folding **4** into a conformation about the NMeAla residue identical to that of *S*-proline in oligopeptide **2**. Thus, we envisaged that **2** and **4** could adopt similar backbone conformations upon binding to the antibody combining site. However, the dissociation constants for the complexes formed between **2**, **3** and **4** and each monoclonal antibody varied considerably. In the case of DB19/1 the variation in dissociation constant was over almost two orders of magnitude. NMR evidence was obtained which indicated that the conformational flexibility of peptides **2**, **3** and **4** was dissimilar in the key tetrapeptide segment. Indeed, amide chemical shift temperature dependence indicated that **3** was significantly less mobile than **2** in this region, presumably due to the additional steric constraints introduced by the methyl group at the C_α carbon of residue 2. Our current interpretation of these results is that the antibodies are extremely sensitive to the entropic component of the binding free energy change, arising from folding the oligopeptide into its bound form. These results are consistent with the view that the proline residue acts only to *restrict* the number of accessible peptide conformations and does not "lock" the molecule into a single conformer in which functional groups are held rigidly in spatial orientations necessary for optimal enthalpic interactions with the antibody.

* Brennand DM *et al.*, manuscript submitted for publication.