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Localization of Tyrosine at the Binding Site of Neurophysin II by Negative Nuclear Overhauser Effects

Sir:

The polypeptide hormones oxytocin and vasopressin are found in neurosecretory granules, in noncovalent association with a group of closely related proteins, the neurophysins.[1] Binding studies using synthetic analogs of the hormones have demonstrated the importance of the α-amino group and the side chains at positions 1–3 in stabilizing the complex;[2–5] tripeptides containing analogs of residues 1–3 display all the principal features of the protein–hormone interaction. We wish to report the results of a 250-MHz proton nmr investigation which further elucidates the molecular details of this binding phenomenon.

Figure 1 shows the proton spectrum of the aromatic ring protons of L-Ala-L-Tyr-L-PheNH$_2$[5] (A) in the presence and absence of bovine neurophysin II (NP-II). The signals from the tyrosine ring are broadened, the protons ortho to the hydroxyl more so than the meta. The phenylalanine ring protons are comparatively unaffected. The tyrosine in position 2 of binding peptides can be substituted by phenylalanine with no significant effect on binding. If (S-Me)Cys-Phe-Ile-NH$_2$ (B) is used as the binding peptide the phenylalanine ring protons show differential broadening, analogous to the tyrosine protons in A. The observations are in agreement with the postulate of rapid reversible binding with specific interactions between the aromatic residue at position 2 of the peptide and unspecified residues on the protein.[3] The differential broadening of adjacent protons on the same aromatic ring suggests a dipolar contribution to the transverse relaxation rate enhancement arising from the proximity of magnetic nuclei on the protein. Such an intermolecular interaction has been considered earlier by Lanir and Navon in their study of the binding of sulfonamide inhibitors to carbonic anhydrase.[6] Strong irradiation of the correct NP-II protons should therefore produce an intensity change, due to an Overhauser effect, in the aromatic resonances from position 2 of the peptide.

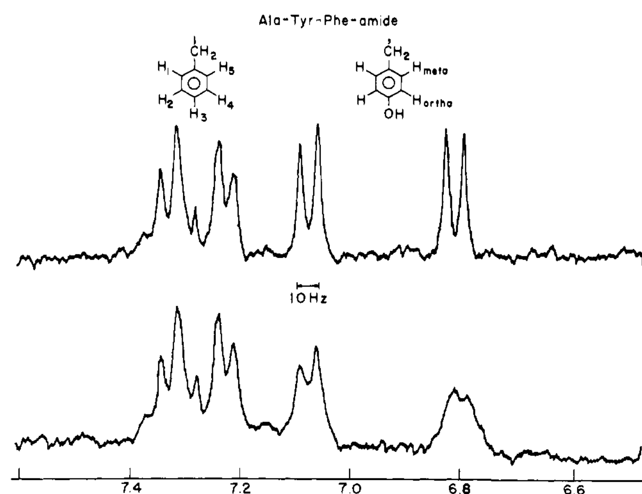

Figure 1. 250-MHz nmr spectrum of the aromatic protons of A: upper trace 2.3×10^{-3} M A in D$_2$O; lower trace, 0.09 equiv of NP-II added, pH 6.5.

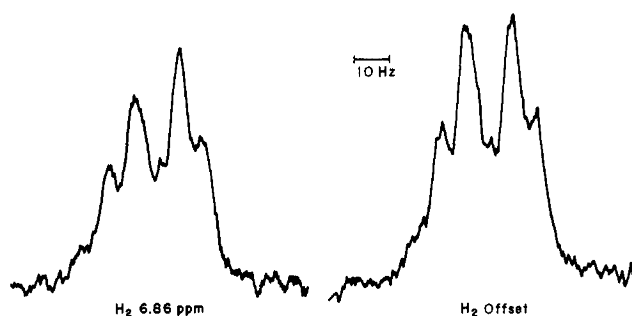

Figure 2. Phenyl protons of **B** in 20:1 mixture of **B** and NP-II with and without strong radiofrequency irradiation at δ 6.86 ppm.

Figure 2 shows the effect on the phenylalanine ring protons of irradiating a 1:20 mixture of NP-II and B at δ 6.86 ppm. A striking decrease in intensity is observed and similar effects were also observed at δ 1.9 and 3.1 ppm. The results on a variety of peptides are summarized in Table I.

Table I. Per Cent Decrease[a] in Intensity of Peptide Signals on Saturating Protein Resonances

Peptide	Resonance obsd[b]	Resonance saturated[g] δ 1.9	δ 3.1	δ 6.86
Ala-Tyr-PheNH$_2$[c]	Tyr ortho	38	36	h
	Tyr meta	14	24	h
(S-Me)Cys-Tyr-PheNH$_2$[d]	Tyr ortho[i]	21	19	h
	Tyr meta	3	9	h
Met-Tyr-PheNH$_2$[e]	Tyr ortho	33	35	h
	Tyr meta	20	29	h
(S-Me)Cys-Phe-IleNH$_2$[f]	Phe 2,3,4	20	18	22
	Phe 1,5	17	15	10

[a] Intensity decreases are crude estimates based on peak heights. There is no change in line width. [b] Refer to Figure 1 for labeling. [c] [Peptide] = 4.5×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 6.5. [d] [Peptide] = 5.4×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 3.5. [e] [Peptide] = 2×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 6.6. [f] [Peptide] = 4.9×10^{-3} M, [NP-II] = 2.3×10^{-4} M, pH 6.5. [g] δ = ppm from DSS. [h] Observing and irradiating frequencies overlap. [i] The NOE's are concentration dependent, a factor disregarded here.

NOE's did not occur in the absence of protein or in the presence of denatured protein. Peptides containing phenylalanine only in position 3 showed no

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NOE's on the phenyl ring protons. Differential NOE's of different protons of the same ring were paralleled by differences in broadening of their resonances. These results unequivocally establish the relationship of the negative NOE to the binding process.

Negative NOE's[7] observed at δ 1.9 and 3.1 ppm cannot be identified with specific alkyl groups on the protein; however, the effect at δ 6.86 ppm can be unambiguously assigned to the ortho protons of the single protein tyrosine residue.[8] These results demonstrate that the protein tyrosine is adjacent to the aromatic ring at position 2 of the peptide, in the complex. This conclusion is supported by preliminary experiments using a neurophysin derivative,[9] in which the single tyrosine is mononitrated. An intensity decrease in the phenyl protons of B in a 14:1 mixture of B and nitro NP-II was observed when the single proton ortho to the nitrotyrosine hydroxyl was saturated ($\delta \sim 6.88$ ppm; pH 6.1). No appreciable effects could be detected on irradiating the proton ortho to the nitro group (δ 7.93 ppm). It should be noted that the chemical shifts of the nitrotyrosine protons are strongly pH dependent in the pH range 6–8 due to the lowered pK_a of the hydroxyl group in the nitrated derivative.

In separate observations of the *protein* tyrosine protons, it was observed that the ortho proton signals became narrower and moved downfield ($\Delta_{\Delta\nu}$ = 6 Hz; $\Delta\delta \approx 20$ Hz) on addition of saturating concentrations of B, indicating that the protein tyrosine is less restricted in the complex than in the free protein. This result suggests that either the protein tyrosine is displaced from an intramolecular binding interaction by the entering peptide, or that it is freed from motional constraints by a local conformational change attendant to binding. In either event the proximity, in the complex, of the aromatic ring at position 2 of the peptide and the protein tyrosine residue, is indicated by the NOE data.

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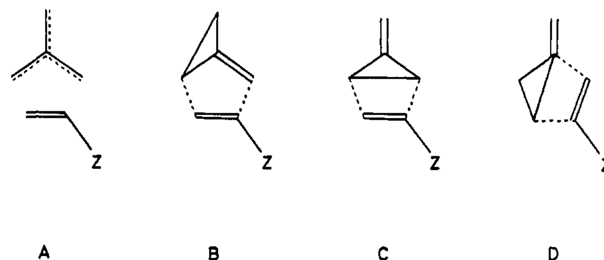
Nickel(0)-Catalyzed Reaction of Methylenecyclopropane with Olefins. Orientation and Stereochemistry[1]

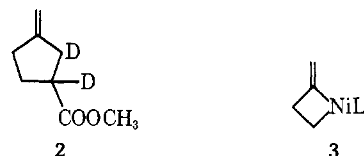
Sir:

Bis(acrylonitrile)nickel(0) ($Ni(AN)_2$) catalyzes the cycloaddition of methylenecyclopropane (**1**) and methyl acrylate yielding 3-carbomethoxymethylenecyclopentane.[2] The new coupling reaction between three- and two-carbon units provides an example of reactions which are *formally* envisaged as thermally forbidden [2 + 2] processes, but which can be realized through metal catalysis. This communication discloses the detailed features of a reaction involving the cleavage of strained carbon–carbon σ bond.

As to the mode of the combination of the two components, four possibilities A–D could be considered *a*



priori (Z = $COOCH_3$). Path A which proceeds *via* trimethylenemethane species (either free or complexed) and path B[3] have been previously eliminated,[2] leaving modes C and D which involve the reaction at the allylic and vinylic bonds of **1**, respectively. The actual orientation of mode D was demonstrated by an examination of the reaction product employing a deuterium-labeled substrate. Reaction of **1** (6 mmol) and methyl acrylate-α,β-d_2[4] (25 mmol) in the presence of $Ni(AN)_2$ (0.2 mmol) under a nitrogen atmosphere (60°, 48 hr) afforded the 1:1 adduct **2** in 70% yield.[5] The structure was determined by nmr analysis (Figure 1). The spectrum of undeuterated 3-carbomethoxymethylenecyclopentane exhibited three-proton, overlapping multiplets at δ 2.4–2.8 arising from methylene protons of C-2 and $CHCOOCH_3$, while the nmr of **2** showed only a one-proton broad signal at the same region.[6] The cycloaddition *via* path D could reasonably be explained by assuming the intermediacy of the organonickel **3** (L =



CH_2 = CHZ) produced by the oxidative addition of the strained carbon–carbon σ bond to the d_{10} Ni(0) atom.[1,7]

The efficiency of the catalyst and the course of the reaction are subtly influenced by the olefinic substrates which act as the metal ligands. Firstly, in the absence of olefins, treatment of **1** with $Ni(AN)_2$ in benzene solution resulted in the recovery of the starting material.[5] Secondly, unlike methyl acrylate which undergoes cyclo-

(1) Nickel-Catalyzed Reactions Involving Strained σ Bonds. IV. Part III: R. Noyori, T. Suzuki, and H. Takaya, *J. Amer. Chem. Soc.*, **93**, 5896 (1971).
(2) R. Noyori, T. Odagi, and H. Takaya, *ibid.*, **92**, 5780 (1970).
(3) For the uncatalyzed [$_\sigma 2 + _\pi 2 + _\pi 2$]-type reaction of methylenecyclopropanes and tetracyanoethylene, see R. Noyori, N. Hayashi, and M. Katô, *ibid.*, **93**, 4948 (1971).
(4) A mixture of cis and trans isomers (87:13 by nmr) prepared by the method of T. Yoshino, J. Komiyama, and M. Shinomiya (*ibid.*, **86**, 4482 (1964)) was used.
(5) A trace amount of 3-cyanomethylenecyclopentane[2] and some butadiene (<5%) were formed.
(6) We thank Dr. T. Nishida of Nippon Electrical Varian, Ltd., for recording the spectra.
(7) J. Halpern, *Accounts Chem. Res.*, **3**, 386 (1970), and references cited therein.