^{13}C NMR STUDIES OF THE CONFORMATION OF *N*-ACETYL-L-TRYPTOPHAN IN ITS COMPLEX WITH CHYMOTRYPSIN

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1. Introduction

N-acyl aromatic amino acids have been termed virtual substrates of chymotrypsin because the oxygen of the carboxyl group is turned over slowly by the enzyme [1]. A detailed picture of the structure of the complex between *N*-formyl-tryptophan and chymotrypsin is available from the crystallographic work of Steitz et al. [2]. Because of possible differences between *N*-formyl substituted compounds and those with larger acylamino groups [3], and because of the importance of the acylamino group as a determinant of the stereospecificity of the enzyme [4], we have now studied the conformation of *N*-acetyl-tryptophan in its complex with chymotrypsin in solution, using ^{13}C NMR.

The particular conformational feature we have examined is the dihedral angle about the C_α–NH bond, since this determines the orientation of the acylamino group relative to the amino acid part of the virtual substrate molecule. It has long been known that there is a relationship between the magnitude of three-bond (vicinal) spin–spin coupling constants and the dihedral angle about the central bond, and the general form of this relationship (the Karplus relationship) is well established, though it differs in detail from one system to another. Bystrov et al. [5, 6] have established the detailed form of the Karplus curve for the coupling constant, $J_{HNC\alpha H}$, between the NH and $C_\alpha H$ protons in amino acids and peptides, and this has been widely used to determine the dihedral angle about the C_α–N bond in peptides in solution. It is not practicable to use this approach in the present system, since the NH and $C_\alpha H$ resonances of the inhibitor will be obscured by the resonances of the many corresponding groups in the enzyme. We have therefore prepared *N*-acetyl[carbonyl-^{13}C]tryptophan, and have used the coupling constant, $J_{CNC\alpha H}$, between the enriched carbonyl carbon and the C_α proton to determine the dihedral angle about the C_α–N bond. Although the dependence of J_{CNCH} on dihedral angle is less well characterised than that of J_{HNCH}, the available data for J_{CNCH} and the closely related J_{COCH} [7–9] are sufficient to obtain an approximate relationship.

2. Materials and methods

N-acetyl[carbonyl-^{13}C] L-tryptophan (90% ^{13}C) was prepared by reaction of L-tryptophan methyl ester with [1-^{13}C] acetic acid (prepared from sodium [1-^{13}C] acetate, Prochem Ltd.) in methylene dichloride in the presence of dicyclohexyl-carbodiimide at 0°C, followed by hydrolysis of the ester in alkali. The product was recrystallised from methanol/water, and characterised by melting point and elemental analysis.

To minimise aggregation δ-chymotrypsin (Sigma Chemical Co.) was used, at concentrations of 3–5 mM in deuterium (^2H_2O) buffered with Tris–HCl, $I = 0.4$, pH 6.9, and containing 8% (v/v) acetonitrile to improve the solubility of the *N*-acetyl-tryptophan, the concentration of which was 10–50 mM.

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^{13}C NMR spectra were obtained at 25.2 MHz with a Varian Associates XL-100-15 spectrometer, operating in the Fourier transform mode. Field/frequency lock was obtained from the $^{2}H_2O$ in the solvent. To simplify the spectrum, and obtain some increase in signal-to-noise ratio by nuclear Overhauser enhancement, selective irradiation at the frequency of the acetyl methyl protons was employed. A pulse interval of 4.00 sec and a spectral width of 500 Hz were used, giving digitisation at 0.1 Hz/point. Between 100 and 13 000 transients were averaged for each experiment.

Since the individual lines of the doublet of the carbonyl carbon resonance overlap quite severely (see fig. 1), the peak separation will be less than the true coupling constant. Two parameters can be measured fairly accurately (± 0.1 Hz) from the spectrum: the peak separation, P, and the total width at half height of the doublet, W_T. These are given by:

$$W_T = W + J$$

and

$$P = f(W, J) \text{ for } W < J$$

where:

W is the width at half height of the individual lines of the doublet;

J is the coupling constant.

The relationship between P, W and J was defined empirically by means of a series of computer simulated spectra, using the line–shape function for two overlapping Lorentzian lines, thus establishing the necessary relationships between the two measured parameters, W_T and P, and the two unknowns W and J.

3. Results

The appearance of the carbonyl carbon resonance at various ratios of substrate concentration to enzyme concentration is shown in fig. 1. It is qualitatively clear that the doublet splitting due to coupling with the α-proton decreases from its value of 2.3 ± 0.1 Hz in the free substrate as the fraction of substrate bound to the enzyme increases. The doublet splitting is observable until the fraction of substrate bound, F, reaches about 0.4, since there is only a moderate increase in line-width of the carbonyl carbon on binding.

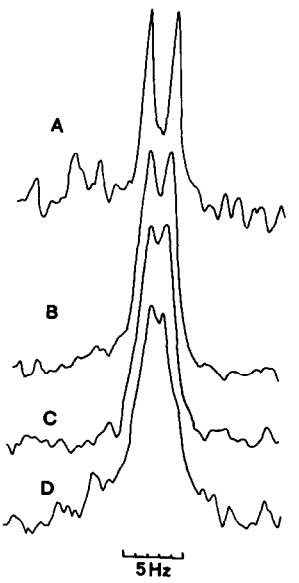

Fig. 1. ^{13}C NMR spectra of NAc[^{13}C] L-Trp bound to δ-chymotrypsin (carbonyl region only shown), pH = 6.84, I = 0.4 (Tris–HCl). The fraction (F) of substrate bound to the enzyme is: A) F = 0.0; B) F = 0.13; C) F = 0.18; D) F = 0.29.

This is presumably due to the fact that the carbonyl carbon does not have a directly bonded proton, and will thus be relatively weakly relaxed.

If the exchange of the N-acetyl-tryptophan molecules between free and bound states is rapid on the NMR time-scale, as seems likely from the appearance of the spectra, and from the value of K_I (2.10^{-3}M; ref. [10]), then the observed coupling constant is given by:

$$J_{obs} = F \cdot J_{bound} + (1 - F) J_{free}$$

where: J_{bound} and J_{free} are the coupling constants characteristic of the bound and free substrate respectively. The value of F for any concentration of substrate and enzyme can be calculated from the binding constant; although this can in principle be obtained from the data in fig. 1, the limited accuracy of the measurements made it preferable to use the value quoted in ref. [10], which was obtained under closely similar conditions.

The coupling constant was determined from the spectra by the method described in Materials and methods; only those spectra in which a distinct doublet splitting could be discerned could be used, so

that values were obtained only for $F < 0.4$. The estimated overall accuracy of the coupling constants is ± 0.4 Hz, though the error is less than this for spectra obtained with only a very small fraction of the substrate bound to the enzyme. The measured coupling constants are shown as a function of F in fig. 2; the line is a least squares fit to the points, and extrapolates to a value of $J_{CNCH} = -0.78 \pm 0.8$ Hz at $F = 1$.

Thus the J_{CNCH} value for *N*-acetyl-L-tryptophan in its complex with chymotrypsin is close to zero, being apparently slightly negative. The experimental data presently available on the relationship between J_{CNCH} and J_{COCH} and dihedral angle [7–9] indicate that the relationship is of the Karplus ($\cos^2\theta$) type, with minima at dihedral angles of ± 90°. The reported values for $\theta \sim 90°$ are very close to zero [7, 9]; theoretical calculations indicate that slightly negative coupling constants might be expected at this point [11]. It seems likely, therefore, that the dihedral angle about the C_α–N bond of *N*-acetyl-tryptophan in the complex is close to ± 90° (corresponding to ϕ [12] = −30 or +150). The combination of the experimental error, and the uncertainty of the exact relationship between J and θ means that this is probably only accurate to ± 20°. In free solution, the value of 2.3 Hz (which compares well with the value of 2.2 Hz reported by Lemieux [9] for Ala–Phe–Gly–[^{13}C]Ala–Ala) would imply $\phi = \pm 50$ or ± 120, but in this case the observed coupling constant is almost certain to be the weighted average of a distribution of conformations.

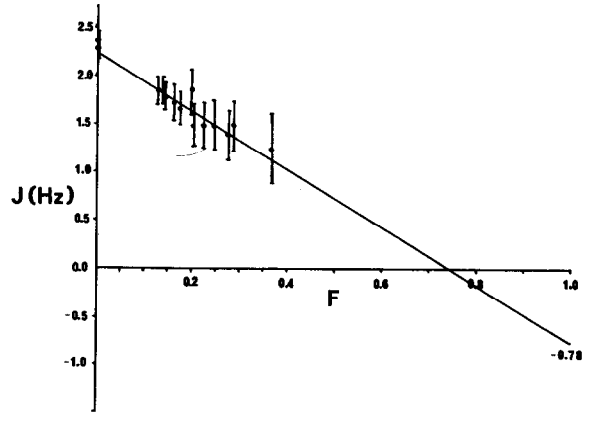

Fig. 2. Plot of observed coupling constant J, against F, the fraction of substrate bound to chymotrypsin, showing least squares best fit line and estimated error in individual observations. The value of J at $F = 1.0$ is − 0.78 (± 0.8) Hz.

4. Discussion

The present experiments represent only the second instance [13] in which measurable changes in coupling constant have been observed for a substrate analogue binding to an enzyme, and illustrate the value of ^{13}C NMR for studies of this sort. The change in coupling constant is quite clear, and shows unambiguously that the conformation of NAcTrp in the complex with chymotrypsin is not the same as its time-average conformation in solution. Though the extrapolated value of the coupling constant in the complex is of limited accuracy, it indicates that the conformation of this virtual substrate when bound to chymotrypsin is such that $\phi \simeq -30°$ or $\simeq +150°$ (the form of the Karplus relationship precludes any distinction between these values). Model-building studies did not indicate any convincing grounds for choosing one of these two alternatives, though simple potential energy considerations [14] would tend to favour $\phi \simeq -30°$.

In the crystallographic study of the *N*-formyl-tryptophan–α-chymotrypsin complex [2], the orientation about the C_α–N bond observed was such that $\phi = -60° \pm 20°$. This is not too different from the value of $-30° \pm 20°$ obtained in the present work. A drawing of the proposed conformation ($\phi = -30°$) of *N*-acetyl-tryptophan in the active site is shown in fig. 3. Steitz et al. [2] suggested that a hydrogen bond between the amide NH and the peptide carbonyl of Ser 214 is responsible for the orientation of the acylamino group, and for the effects of modifications in this group on the rate of the enzymatic reaction [3, 4]. Some uncertainty on this point remains however, since the enzyme crystallises with two molecules in the asymmetric unit, related by a dyad axis which passes close to the active site region of the molecule. In particular, the acylamino NH of the *N*-formyl-tryptophan approaches within 3.2 Å of the phenolic hydroxyl of Tyr 146 of the dyad-related molecule, raising the possibility that it is this interaction and not the NH–Ser 214 one which is responsible for the orientation of the acylamino group.

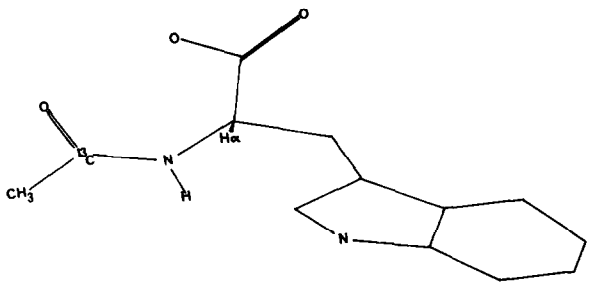

Fig. 3. The $0\bar{1}1$ projection of the conformation of NAc L-Trp in the active site of chymotrypsin (co-ordinate reference frame defined by Birktoft et al. [17]).

There are two pieces of evidence which suggest that the presence of the dyad-related molecule does not seriously distort the orientation of the substrate at the active site. First, the crystals are enzymatically active toward small substrates [15] and secondly, model-building experiments with pancreatic trypsin inhibitor [16] give further evidence for an NH–CO (Ser 214) hydrogen bond. Since the present NMR results are at least approximately consistent with the crystallographic data, one can conclude that any differences in the orientation about the C_α–N bond of these virtual substrates between crystal and solution are minimal.

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