N.M.R. Studies of Enzyme Mechanism. Comparison of the Crystal Structure and Solid State ^{13}C and ^{15}N N.M.R. Spectra of a Carboxypeptidase A Complex with Glycyl Tyrosine

Neil E. Mackenzie, Paul E. Fagerness, and A. Ian Scott*

Center for Biological N.M.R., Department of Chemistry, Texas A&M University, College Station, Texas 77843, U.S.A.

It is shown that solid state n.m.r. spectroscopy can be used to determine the extent of cleavage of the scissile amide bond in glycyl tyrosine, a slow substrate for the enzyme carboxypeptidase A in the crystalline enzyme substrate complex.

The use of high-field n.m.r. spectroscopy to study enzyme mechanism in solution has become a powerful technique for the establishment of the structures of productive, covalently bound intermediates.[1] The basis for these studies has almost invariably relied on the crystal structure determination of the enzyme and/or enzyme complex at the active site. Since the phase change from solid to solution may well involve considerable conformational alteration, the correlation of the two physical methods (*X*-ray, n.m.r.) in the same phase would be a desirable objective in furthering our understanding of enzyme mechanism. We now describe the application of solid state cross polarization magic angle spinning (CPMAS) n.m.r. spectroscopy to the direct observation of the hydrolysis of a slow (pseudo) substrate catalysed by an enzyme. Magic angle

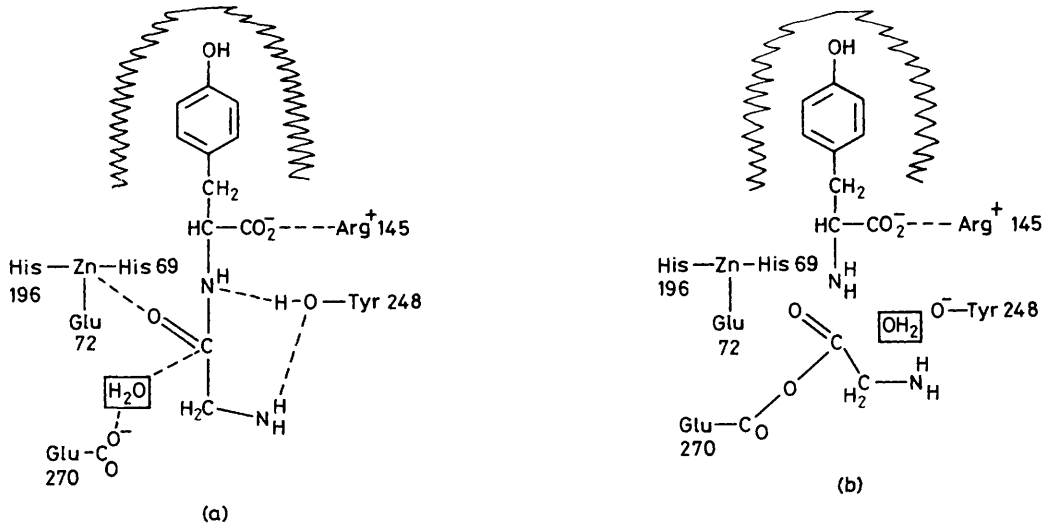

Figure 1. (a) Gly-Tyr bound to CPA showing the indirect attack of Glu-270 promoting the attack of a water molecule on the substrate's amido carbonyl group polarized by interaction with zinc. (b) Direct attack of Glu-270 on the substrate's amido carbonyl forming an anhydride.

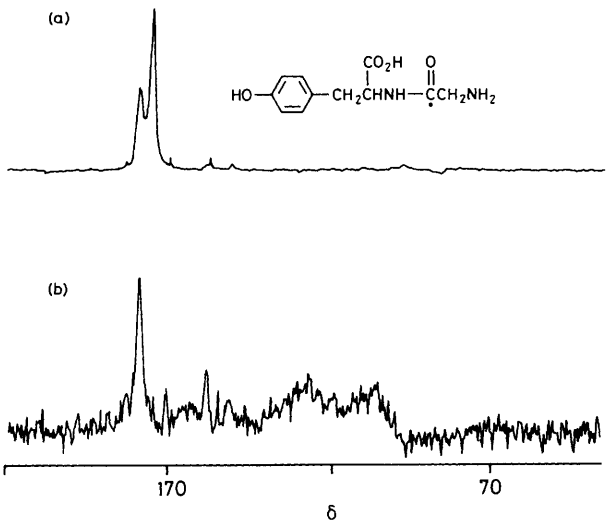

Figure 2. 25.15 MHz Solid state ^{13}C CPMAS spectrum of (a) amido-[^{13}C] Gly-Tyr, ● = ^{13}C, and (b) the difference CPMAS spectrum of CPA and CPA/amido-[^{13}C] Gly-Tyr complex. All spectra are 4K data points with 10 Hz line broadening; (a) represents 1076 scans and (b) is the weighted difference of 58 027 scans and 50 225 scans.

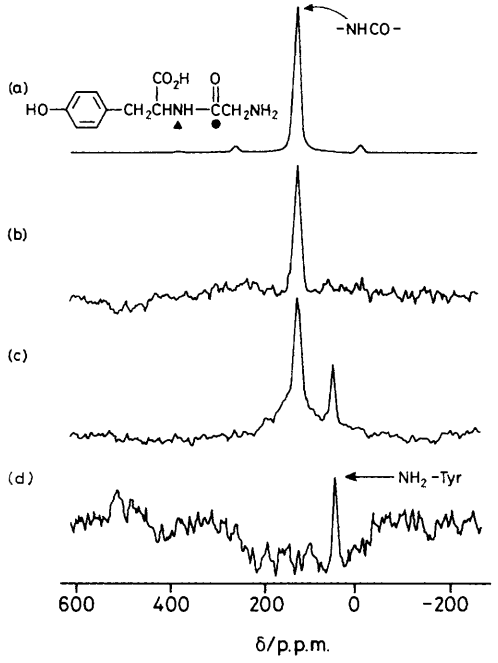

Figure 3. 20.28 MHz Solid state ^{15}N CPMAS spectrum of (a) amido-[^{13}C, ^{15}N] Gly-Tyr, ● = ^{13}C, ▲ = ^{15}N, (b) CPA, and (c) CPA + amido-[^{13}C,^{15}N] Gly-Tyr. All spectra are 1K data points with 100 Hz line broadening; (a) represents 8 884 scans, (b) 80 000 scans, (c) 80 000 scans, while (d) represents the difference between (b) and (c). The ^{15}N resonance shown in (b) results from the presence of *ca.* 300 natural abundance amido nitrogens. A similar resonance in the ^{13}C CPMAS n.m.r. spectrum (not shown) was observed for the complementary *ca.* 300 amido carbons. *N.b.* 1 enriched centre = *ca.* 100 unenriched (depending on level of enrichment).

spinning (MAS) n.m.r. has already been shown to be a powerful technique for the study of solids generally[2] and has been used extensively on inorganic systems.[3]

The crystal structures of several complexes of the metalloenzyme, carboxypeptidase Aα (E.C. 3.4.17.1) (CPA), have been examined in considerable detail. The structure of the complex with glycyl tyrosine (Gly-Tyr) has been refined to 2.0 Å resolution[4,5] and reveals *inter alia* interactions between the amide carbonyl oxygen and the catalytically essential zinc, and between the amide nitrogen and the hydroxy group of tyrosine-248 (Tyr-248), Figure 1(a). The proposed mechanisms for hydrolysis of peptide and ester bonds by CPA have relied heavily on these crystal structures, but a clear distinction between the possible roles of glutamate-270 (Glu-270) in nucleophilic attack either by general base catalysis, Figure 1(a), or by covalent anhydride formation, Figure 1(b), remains a major unresolved problem. Indeed, it is not yet certain whether esters and amides are hydrolysed by CPA *via* identical mechanisms.[6]

Gly-Tyr was synthesized as both the amido-[^{13}C] (90 atom % enrichment) and amido-[^{13}C,^{15}N] (90 atom % and 99 atom % enrichment, respectively) isotopomers by known methods.[7] CPA, purchased from Sigma as a suspension in toluene, was crosslinked[8] and soaked with Gly-Tyr as described previously[4] and the washed, dried crystals examined by solid state n.m.r. spectroscopy. The ^{13}C CPMAS n.m.r. spectrum of the ^{13}C enriched Gly-Tyr, Figure 2(a), displays residual[9] ^{13}C–^{14}N quadrupolar coupling (δ 176). However, the ^{13}C CPMAS difference spectrum of CPA and the CPA/Gly-Tyr complex, Figure 2(b), displays a *single resonance* (δ 178, $w_{\frac{1}{2}}$ 60 Hz) revealing that, under the experimental conditions, the peptide bond of this slow reacting substrate has been cleaved to the extent of >90%. In order to confirm this result, doubly labelled amido-[^{13}C,^{15}N] Gly-Tyr, Figure 3(a), was bound to CPA under identical conditions and the complex examined by ^{15}N CPMAS n.m.r. spectroscopy. As can be seen from Figure 3(a), the ^{15}N resonance of the substrate (δ 120 p.p.m.) is sufficiently broad ($w_{\frac{1}{2}}$ 325 Hz) to conceal the ^{15}N–^{13}C one bond scalar coupling (J 16 Hz). In the enzyme complex, Figure 3(c), the observed ^{15}N resonances correspond to the amide region of CPA [see Figure 3(b); δ 122 p.p.m., $w_{\frac{1}{2}}$ 400 Hz] and an amine[10] (δ 42 p.p.m., $w_{\frac{1}{2}}$ 250 Hz), respectively. We infer from these experiments that we are observing *either* the first stable intermediate of the catalytic pathway, *i.e.* the anhydride of the cleaved glycine residue bound covalently to Glu-270, Figure 1(b), or the *bound glycine*. In either case there is a second product, tyrosine [Figure 3(d)] also bound to CPA, since in a separate experiment it could be shown that no unbound tyrosine or glycine was present in the complex. A distinction between the two glycine species (anhydride or acid) cannot be made at this stage since the ^{13}C chemical shifts of the carbonyl(s) in both the anhydride and the acid are within the observed chemical shift range. However, these preliminary experiments clearly indicate the feasibility of simultaneous X-ray and CPMAS n.m.r. studies, especially with slow substrates. In summary, a method of some generality for reaching the long-sought correlation between X-ray diffraction data, the resultant geometry of a substrate undergoing slow catalysis, and the corresponding n.m.r. chemical shifts for selected atoms in the *enriched* substrate, obtained under identical conditions, is now at hand.

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References

1 N. E. Mackenzie, J. P. G. Malthouse, and A. I. Scott, *Science*, 1984, **225**, 883.
2 E. R. Andrew, *Int. Rev. Phys. Chem.*, 1981, **1**, 195.
3 C. A. Fyfe, J. M. Thomas, J. Klinowski, and G. C. Gobbi, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 259.
4 W. N. Lipscomb, J. A. Hartsuck, G. N. Reeke, Jr., F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, 1968, **21**, 24; W. N. Lipscomb, *Acc. Chem. Res.*, 1982, **15**, 232.
5 W. N. Lipscomb, G. N. Reeke, Jr., J. A. Hartsuck, F. A. Quiocho, and P. H. Bethge, *Philos. Trans. R. Soc. London, Ser. B*, 1970, **257**, 177.
6 W. N. Lipscomb, in 'Proceedings of The Robert A. Welch Conferences on Chemical Research. XV. Bioorganic Chemistry and Mechanisms,' 1971, p. 131; R. Breslow, J. Chin, D. Hilvert, and G. Trainor, *Proc. Natl. Acad. Sci. USA*, 1983, **80**, 4585; S. J. Hoffmann, S. S.-T. Chu, H. Lee, E. T. Kaiser, and P. R. Carey, *J. Am. Chem. Soc.*, 1983, **105**, 6971; L. C. Kuo, J. M. Fukuyama, and M. W. Makinen, *J. Mol. Biol.*, 1983, **163**, 63.
7 J. P. Greenstein and M. Winitz, in 'Chemistry of the Amino Acids,' Wiley, New York, 1961.
8 F. A. Quiocho and F. M. Richards, *Biochemistry*, 1966, **5**, 4062.
9 M. H. Frey and S. J. Opella, *J. Chem. Soc., Chem. Commun.*, 1980, 474.
10 G. C. Levey and R. L. Lichter, in 'Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy,' Wiley, New York, 1979.