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Studying Enzyme Mechanism by ^{13}C Nuclear Magnetic Resonance

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Summary. High-resolution carbon-13 nuclear magnetic resonance (NMR) spectra of enzyme-inhibitor and enzyme-substrate complexes provide detailed structural and stereochemical information on the mechanism of enzyme action. The proteases trypsin and papain are shown to form tetrahedrally coordinated complexes and acyl derivatives with a variety of compounds artificially enriched at the site or sites of interest. These results are compared with the structural information derived from x-ray diffraction. Detailed NMR studies have provided a clearer picture of the ionization state of the residues participating in enzyme-catalyzed processes than other more classical techniques. The dynamics of enzymic catalysis can be observed at sub-zero temperatures by a combination of cryoenzymology and carbon-13 NMR spectroscopy. With these powerful techniques, transient, covalently bound intermediates in enzyme-catalyzed reactions can be detected and their structures rigorously assigned.

In recent years nuclear magnetic resonance (NMR) spectroscopy has been used extensively to study the mode of action of various enzymes with both inhibitors and substrates. So far, reporter nuclei, including ^{15}N, ^{13}C, ^{31}P, and ^{1}H, have been observed either at or near the active site of several enzymes. These studies have been reviewed elsewhere (*1–3*) and will be referred to in our discussion, which is concerned with the reactions of ^{13}C-enriched inhibitors and substrates with enzymes. Until recently, ^{13}C has not received much attention in relation to enzyme mechanism, in part because of certain inherent disadvantages. These include a low natural abundance (1.1 percent) compared to ^{31}P (100 percent) and ^{1}H (100 percent) and a relative insensitivity to the NMR experiment resulting from the small gyromagnetic ratio (μ) of ^{13}C, which directly governs the size of the nuclear response to electromagnetic excitation (*4*). To overcome these problems, the site or sites of interest in any system must be artificially enriched (either synthetically or biosynthetically) in ^{13}C to levels approaching 100 percent. The ultimate benefit from such tedious and time-consuming operations is that ^{13}C becomes the nucleus of choice in monitoring enzyme-substrate (or enzyme-inhibitor) interactions since its wide range of chemical shifts (≈220 ppm) makes it an exquisitively sensitive probe of molecular structure.

With few exceptions, the use of ^{13}C NMR spectroscopy in studying enzyme reactions has been directed toward monitoring the fate of a specifically enriched carbonyl function in a substrate or inhibitor. This is a consequence of the large chemical shift change (Δ) brought about by substitution α to this group (Δ ~2 to 30 ppm) or by direct covalent attachment of the carbonyl (Δ ~100 ppm) to the enzyme. There are other advantages of the carbonyl function (and quaternary carbons in general). (i) Full nuclear Overhauser effect (*5*) proton decoupling can be maintained by low decoupler settings with concomitant minimization of dielectric heating. (ii) Since chemical shift anisotropy (CSA) is the predominant relaxation mechanism at high field strengths, the values of the longitudinal relaxation times are reduced (*6*), allowing rapid data acquisition without saturation. (iii) The line widths of the observed signals convey important information (see below) and are smaller for quaternary carbons relaxing by the CSA mechanism than for carbons having directly bonded protons, which have a predominantly dipolar relaxation mechanism (*6, 7*). The gain in sensitivity resulting from the use of very high magnetic fields (up to 500 MHz) is offset to some extent by increased line width at high field strength.

Almost all enzymatic processes utilize multistep reactions during catalysis, and the characterization of each of these stages is essential if one is to understand enzyme mechanism at the molecular level. Earlier investigators have used spectrophotometric methods for characterizing enzyme-catalyzed reactions, whereas inhibitors that form stable "transition state analogues" have been examined by other techniques that require long periods of data accumulation, for example, x-ray analysis. However, the advent of NMR and its subsequent use in enzymology are beginning to provide a novel and penetrating probe for elucidating enzyme mechanism by directly characterizing intermediates formed in catalysis. The studies of "transition state analogues" allow access to the unique properties of enzymes that enable them to stabilize labile intermediates and therefore achieve their remarkable catalytic efficiency. We discuss first the structures of enzyme-inhibitor adducts of three examples of proteases—trypsin, papain, and

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carboxypeptidase—and compare the results of direct observation by ^{13}C NMR with the structural information inferred by more classical techniques. We then review the powerful combination of NMR and cryoenzymology. The development of this technique, whereby enzyme-catalyzed reactions are studied at sub-zero temperatures in aqueous organic solvents (cryosolvents), allows such reactions to be slowed down and can prolong the lifetime of enzyme-substrate intermediates (*8, 9*).

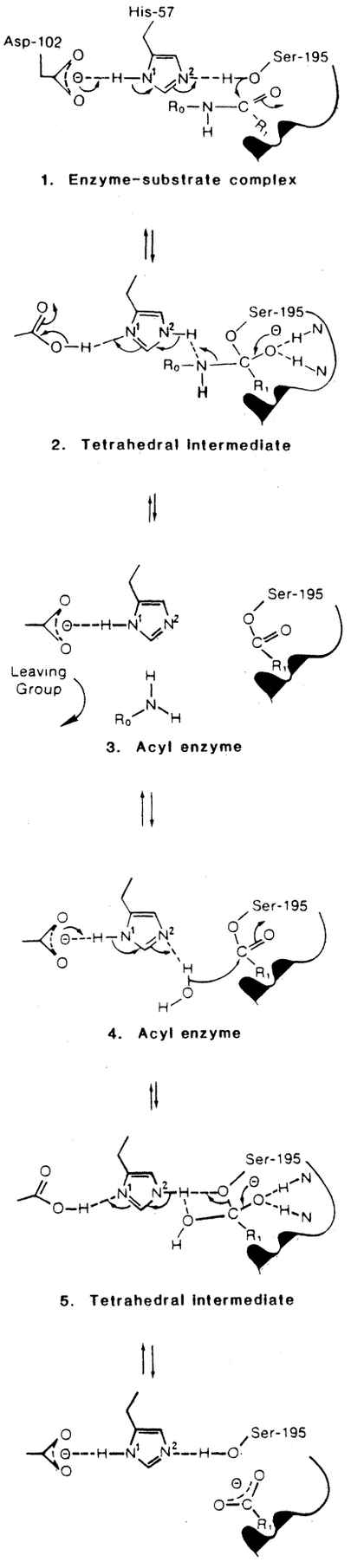
Proteases

The proteases are classified according to the nature of their functional groups. For example, the thiol and serine proteases have reactive cysteine thiol and serine hydroxyl groups, respectively, whereas the acid proteases have a catalytically essential carboxylate group. The latter function (and others) are combined with metal (for example zinc) in the carboxypeptidases, which are metalloenzymes. It is generally assumed that, for both the thiol and serine proteases, catalysis proceeds via tetrahedral and acyl intermediates as shown in Scheme 1 (*10*). As we shall see later, ^{13}C NMR allows the direct observation, identification, and characterization of such species, providing direct confirmation of the structure and existence of such intermediates in enzyme catalysis.

Scheme 1 shows the generally accepted mechanism for the hydrolysis of an amide function by a serine protease and is representative of the mechanism for all the hydrolyses except that the active-site nucleophile OH of serine (see **1** in Scheme 1) can be replaced by the groups described above. To confirm this mechanistic pathway, the visualization and rigorous characterization of productive tetrahedral intermediates and acyl enzymes by ^{13}C NMR is the ultimate goal, and we now discuss the progress made so far, particularly with the serine and thiol proteases.

Serine Proteases: Inhibitor Complexes of Tetrahedral Geometry

Henderson (*11*) was the first to suggest that the tetrahedral intermediates (**2** and **5** in Scheme 1) are specifically stabilized at the active site of a serine protease via hydrogen bonding of this anionic species. Although transition-state stabilization (*12*) of a tetrahedral intermediate may account for much of the catalytic efficiency of proteases, the stoichiometric accumulation of such a species would not result in efficient catalysis. Hence, the detection of such an intermediate during catalysis is unlikely under normal conditions of physiological *p*H and temperature.



Scheme 1.

Inhibitors that have the potential to form tetrahedral adducts with the thiol and serine proteases, and to behave as transition state analogues (*13, 14*), include both synthetic and natural inhibitors usually containing a carbonyl group at the scissile position. For example, the potency of both soybean trypsin inhibitor (STI) and pancreatic trypsin inhibitor (PTI) was first attributed to their ability to bind as tetrahedral adducts of trypsin at the scissile C=O center (*15*). However, later x-ray crystallographic evidence (*16*) suggested that a tetracovalent inhibitor complex was not formed, although the refined data indicated distortion of the bound C=O group from planarity. The latter view was confirmed by ^{13}C NMR experiments in which both STI and PTI were prepared with the appropriate carbonyl carbon enriched in ^{13}C and neither showed any significant perturbation (<1.0 ppm) of the carbonyl resonance in the inhibitor complex with trypsin (*17–19*). A fully tetrahedral adduct would have displayed an upfield shift (Δ) of ~100 ppm.

In the domain of synthetic inhibitors, chloromethylketone derivatives of specific substrates are potent irreversible covalent inhibitors of the serine proteases, alkylating the active-center histidine at N-2 (see Schemes 1 and 2). X-ray crystallographic studies (*20*) led to the suggestion that, in addition to the above alkylation, there was also nucleophilic attack by the active-center serine hydroxyl to form a hemiketal, which is stereochemically analogous to the tetrahedral intermediate purported to occur during catalysis.

In ^{1}H NMR studies (*21*) of chymotrypsin inhibited by chloromethylketones, attempts have been made to characterize the hydrogen bond between the histidine and aspartate residues (Scheme 1) involved in the catalytic triad of these enzymes (Ser-195, His-57, and Asp-102). These studies showed that the exchange rate of the N-1 proton and the chemical shift upon *p*H titration are both reduced relative to the native enzyme. It was also calculated that the pK_a (negative logarithm of the acidity constant) of this proton was ≈8.4, possibly reflecting ionization of a tetrahedral adduct, with the histidine-aspartate hydrogen bond remaining intact (Scheme 2). Alkylation of N-2 would be expected to raise the pK_a of the active-center histidine

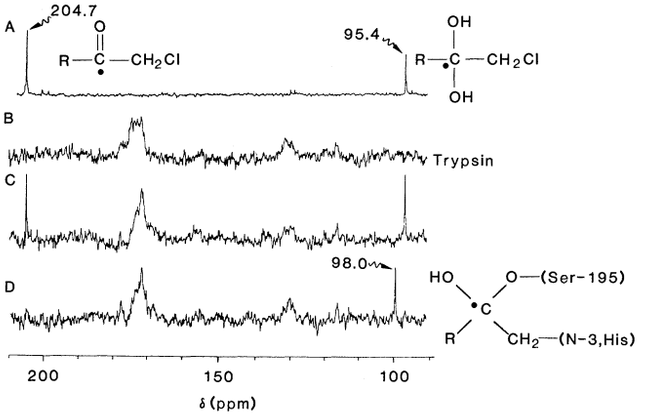
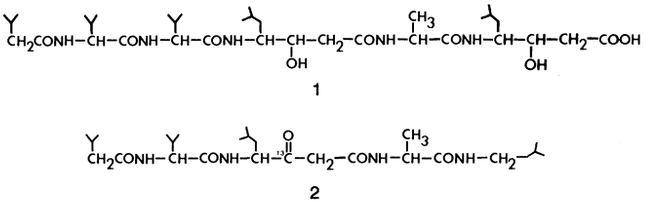


Fig. 1 (left). The ^{13}C NMR spectra of (A) N^{α}-carbobenzyloxylysylchloromethylketone ($RCOCH_2Cl$); ● = ^{13}C, 90 percent enrichment; 47.6 m*M* (by weight); 1 m*M* HCl; D_2O, 16.7 percent (by volume); volume, 0.6 ml; 1660 accumulations; *p*H 3.1; (B–D) 20 m*M* sodium phosphate; 12.5 percent (by volume) D_2O; 10,000 accumulations; trypsin, 0.28 m*M* (concentration of fully active enzyme); volume, 8 ml. The $RCOCH_2Cl$ concentrations, *p*H, and enzyme activities were as follows: (B) 0.00 m*M*, 3.2, 100 percent; (C) 0.38 m*M*, 3.2, 100 percent; (D) 0.37 m*M*, 6.9, 0.06 percent. All spectra were accumulated on a Bruker WM300 WB spectrometer at 75.4 MHz for ^{13}C nuclei.



1

2

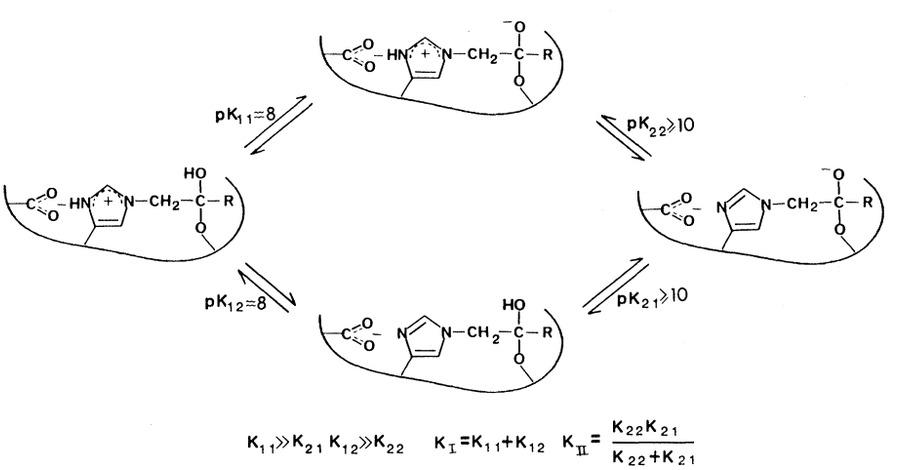
Fig. 2 (right). Structure of pepstatin (**1**) and a synthetic analogue (**2**) enriched in ^{13}C in the ketonic function.

(pK_a >10?) as the N-1 proton is no longer freely available to solvent.

To test these suggestions trypsin was inhibited with the highly specific reagent N^{α} - carbobenzyloxylysylchloromethyl - ketone (*22*) ($RCOCH_2Cl$), labeled in the ketone carbon with 90 percent ^{13}C, for it was predicted that a tetrahedral adduct, if formed, would be directly observable by this technique. In aqueous solution (Fig. 1A), $RCOCH_2Cl$ exists as a mixture of the ketone (δ = 204.7 ppm) and its hydrate (δ = 95.4 ppm) (*23*). At *p*H 3.2 no alkylation or inhibition of the enzyme is observed and the spectrum of [^{13}C=O]$RCOCH_2Cl$ is unperturbed (Fig. 1, B and C), showing that at low *p*H there is no detectable binding or tetrahedral adduct formation prior to alkylation. At *p*H 6.9 there is rapid, irreversible inhibition, and resonances due to both [^{13}C=O]$RCOCH_2Cl$ and its hydrate are replaced by a single resonance at 98.0 ppm (Fig. 1D), an indication that the ^{13}C-enriched carbonyl of the inhibitor is tetrahedrally coordinated in the inhibitor-enzyme adduct. Denaturation (*23*) of the trypsin led to reappearance of a carbonyl resonance (205.5 ppm) and a decrease in the intensity of the resonance at 98.0 ppm. This demonstrates that the tetrahedral adduct formed by the attack of the serine hydroxyl on the inhibitor carbonyl and characterized by the resonance at 98.0 ppm requires an intact trypsin structure.

The chemical shift (98.0 ppm) of the tetrahedral adduct increased with increasing *p*H (pK_a 7.9) to 102.1 ppm. Studies with model compounds (*24*) suggest that the β-shift observed on deprotonation of alkylated imidazoles is too small to account for the Δ of the ^{13}C-enriched carbon in the enzyme adduct. A pK_a of 7.9 is in reasonable agreement with that (8.4) described in the ^1H NMR studies of Robillard and Shulman (*21*) and suggests that this pK_a (pK_{11} in Scheme 2) does indeed reflect the ionization of a tetrahedral adduct (Scheme 2). It has also been found that with negatively charged inhibitors (*25*) the histidine-aspartate hydrogen-bonded proton does not titrate; this has led to the suggestion (*26*) that possibly the tetrahedral adduct can be stabilized by interaction with the protonated histidine. Such an interaction would explain the low pK_a value of the tetrahedral adduct and would also raise the pK_a of the active-center histidine, normally at a value of ≈7. A small fraction of the histidine would therefore ionize with a pK_a (K_{12} in Scheme 2) of ≈8 and the remainder (K_{22} in Scheme 2) with a pK_a >10, while conversely a small fraction of the tetrahedral adduct would ionize (K_{21} in Scheme 2) with a pK_a >10 and the remainder with a pK_a (K_{11} in Scheme 2) of ~8. Incorporation of ^{13}C-enriched histidine (at the C-2 of the imidazole ring) into α-lytic protease has shown that in the native enzyme the histidine is protonated with a pK_a of ~7 (*2, 27, 28*). However, in the presence of an aldehydic inhibitor the chemical shift of the carbon-enriched imidazole (of histidine) titrates cooperatively with pK_a values of 7 and 5.5 and the histidine is neutral at *p*H 8.0 (*29*). Fastrez (*30*) has argued that this suggests that an anionic tetrahedral adduct is not stabilized by interaction with the protonated histidine. It has, however, been shown by x-ray crystallography (*31*) that aldehydes binding to α-lytic protease cause large movements of the active-center histidine, which is consistent with the cooperative ionization observed in the ^{13}C NMR study by Hunkapillar *et al.* (*29*). In chloromethylketone inhibitor complexes and presumably in true enzyme-substrate complexes in which the leaving group prevents such movement, histidine-57 is held firmly in place.

In the above experiments with [^{13}C=O]$RCOCH_2Cl$ and trypsin, it is difficult to discount the possibility that the resonance at 98.0 ppm could result



Scheme 2. K_I and K_{II} are the experimentally observed molecular dissociation constants, which are related to the group dissociation constants (K_{11}, K_{12}, K_{21}, and K_{22}).

from hydration of the carbonyl of the covalently bound inhibitor. Clearly, however, both the neutral and the ionic tetrahedral species are stabilized by trypsin, as no resonance near 205 ppm is detectable (*23, 32*). This confirms that trypsin can stabilize both the neutral and anionic tetrahedrally coordinated adducts.

The line width of the resonance at 98.0 ppm in the *p*H range 3.75 to 7.0 (7 to 10 Hz) is close to the value expected for a quaternary carbon at 75 MHz on a protein of molecular weight 24,000 (*6*).

Papain and Pepsin

N-acetylphenylalanylglycinal is an extremely potent inhibitor of papain (*14*) and is presumed to form stable hemithioacetals which resemble tetrahedral intermediates. Inhibition of papain with *N*-acetyl-L-phenylalanyl[1-^{13}C]glycinal (δ = 200.9 ppm) has made possible the direct observation of hemithioacetal formation by ^{13}C NMR spectroscopy (*33*). The lower electronegativity of sulfur compared to oxygen results in decreased deshielding of the enriched carbon in the thiohemiacetal relative to the hydrated aldehyde. As a result, the papain thiohemiacetal resonance at δ = 75 ppm is clearly resolved from the hydrate resonance (δ = 88.2 ppm). Earlier ^{1}H NMR studies (*34, 35*) involving aldehydic inhibition of papain showed by cross-saturation that indeed the free aldehyde and not the hydrate is the inhibiting species of this reaction. Scale expansion of the signal at 75 ppm revealed that it was composed of two resonances, one at 75.02 ppm and another at 74.68 ppm. The former resonance can be selectively removed by titration of the free enzyme with 2,2′-dipyridyldisulfide (*36*) at *p*H 7.0. This allowed the ^{13}C-^{1}H coupling constant (*J*) values to be assigned as 155 Hz and 160 Hz for the low-field and high-field signals, respectively. Both the chemical shift values and the separation of the papain hemithioacetal resonances are very similar to those of the diasteroisomeric hemithioacetals formed chemically from *N*-acetyl-L-phenylalanyl[1-^{13}C]glycinal and L-cysteine, which display resonances at 75.8 and 76.8 ppm.

The line widths of the papain thiohemiacetal resonances are approximately 50 Hz, from which, if we assume predominantly dipolar relaxation, the average rotational correlation time (τ_r) is estimated at 36 nsec. This value lies in the range expected for a protein of molecular weight 23,406; for example, myoglobin, molecular weight 17,600, τ_r = 19 nsec; hemoglobin, molecular weight 64,500, τ_r = 47 nsec (*7*). The aldehyde inhibitor is therefore rigidly held to the protein with no significant librational freedom. These results illustrate the value of ^{13}C NMR spectroscopy as a direct stereochemical probe of enzyme-inhibitor adducts. The fact that only one diastereoisomer is observed in the tetrahedral adduct formed with the ketonic inhibitor adducts of trypsin (*23*) indicates a much greater enzymatic stereospecificity in this case.

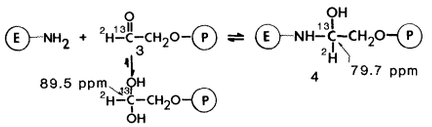


Fig. 3. Reaction of rabbit muscle aldolase with [1-^{2}H, 1-^{13}C]hydroxyacetaldehyde phosphate (**3**) to form a carbinolamine adduct (**4**); P, phosphate; E, enzyme.

A similar situation has been found in inhibition studies of pepsin, an acid protease. Pepstatin (isovaleryl-Val-Val-Sta-Ala-Sta) (**1** in Fig. 2) is a specific and potent inhibitor ($K_I = 5 \times 10^{-11} M$) (*37, 38*) of this enzyme, and its effectiveness led to the suggestion that the 3*S*-hydroxyl of the internal statin residue acts as an analogue of a tetrahedral intermediate (*39, 40*) formed during catalysis.

A ketone analogue (**2** in Fig. 2) of pepstatin was also found to be an effective inhibitor of pepsin (*41*) ($K_I = 5.6 \times 10^{-9} M$) and was prepared with the ketonic function enriched in carbon-13 [δ = 204.2 ppm; $CHCl_3$-d (85 percent); CH_3OH-d (15 percent)] (*42*). On inhibition of pepsin with structure **2** in Fig. 2 a new single resonance at 99 ppm was observed and assigned to a tetrahedral adduct. Evidence that this resonance arises from an enzyme-bound species was derived by addition of the more effective inhibitor, pepstatin, which caused the resonance at 99 ppm to disappear and to be replaced by one at 204.2 ppm. As in the case of trypsin, the remote possibility remains that the enzyme could specifically stabilize the ketone hydrate, although the spectrum of ketone displaced by pepstatin gave no evidence of a hydrate resonance.

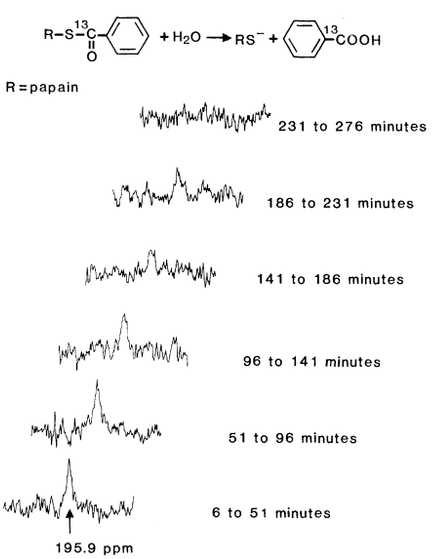


Fig. 4. Reaction of 1.7 m*M* papain (72 percent active enzyme), 5.4 m*M* potassium chloride, 25 percent (by volume) dimethyl sulfoxide, 0.1*M* sodium formate buffer (*p*H 4.1), and 23.6 m*M* benzoylimidazole. [^{13}C=O]Benzoylimidazole was added at 0°C; after 15 minutes the reaction was cooled to −6°C, and the NMR data acquisition commenced 6 minutes after the reaction was initiated. Spectra represent 10,000 accumulations recorded sequentially starting 6, 51, 96, 141, 186, and 231 minutes after adding benzoylimidazole (*48*). The spectral range shown is 192 to 200 ppm.

Carboxypeptidase

A recent ^{13}C NMR study (*43*) has been performed on this last subset of the proteases to establish the distribution of charge in the enzyme-inhibitor complex formed between carboxypeptidase A and the powerful reversible inhibitor 2-benzylsuccinate. To this end, the inhibitor was enriched with ^{13}C in the two carboxyl groups, alternatively. Thus, upon inhibition with both 2-benzyl[1-^{13}C]succinate and 2-benzyl[4-^{13}C]succinate, in two separate experiments, these carboxylate resonances remained intact in the inhibition complex although moved to lower fields. Titration of these resonances showed that they were both ionized in the bound inhibitor complex. However, from the magnitude of the chemical shift in the case of inhibition with 2-benzyl[4-^{13}C]succinate it was inferred that one of the oxygens of the 4-carboxylate group is in coordination with the active-site zinc and that the other projects directly toward the protonated γ-carboxylate group of Glu-270 and forms a hydrogen bond.

Aldolase: Detection of a Carbinolamine

Schiff base formation between the ϵ-amino group of a lysine residue of an enzyme and the carbonyl group of a substrate is common, for example, in fructose diphosphate aldolase (*10*), which mediates the condensation of dihydroxyacetone phosphate with glyceraldehyde phosphate. However, ^{13}C NMR studies (*44*) with the pseudosubstrates [^{13}C=O]hydroxyacetone phosphate and [^{13}C=O, ^{2}H]hydroxyacetal-

dehyde phosphate (**3** in Fig. 3) showed that the former binds noncovalently (chemical shift differences of ≤ 0.1 ppm) and, most interestingly, that the latter forms a carbinolamine adduct (**4** in Fig. 3) (δ = 79.7 ppm) at the active site.

The Quest for True Intermediate Structure: Cryoenzymology

The detection and characterization of a productive acyl intermediate by ^{13}C NMR during catalysis with natural substrates at ambient temperatures is not possible at present, because there is an inherent lack of sensitivity in ^{13}C NMR spectroscopy. Therefore, the lifetimes of these intermediates must be extended into the domain of the NMR experiment. One approach is to use synthetic substrate analogues which deacylate slowly. Another is to utilize low-temperature cryoenzymological techniques to extend the lifetimes of intermediates.

For example, nonspecific dithioester substrates give rise to dithioacylpapains that have been directly observed by ultraviolet (*45*) and resonance Raman spectroscopy (*46*). These dithioacylpapains are thought to be analogous to the thioacylpapains that are believed to occur during catalysis of natural ester substrates. The acylation of papain by *N*-cinnamoylimidazole causes the ultraviolet spectrum to be red-shifted by 20 nm relative to the model (*S*)-*trans*-cinnamolycysteine, and only on denaturation of the "acylated papain" did the spectrum resemble that of the model compound (*47*).

Since the ^{13}C resonances of the carbonyl carbon of thioesters are shifted (Δ 20 to 30 ppm) upfield relative to their oxygen analogues ($\delta \approx 165$ to 185 ppm), ^{13}C NMR spectroscopy should allow the direct monitoring of the formation and decay of a thioacyl intermediate. Using [^{13}C=O]*N*-benzoylimidazole (δ = 168.7 ppm), we were able to observe directly a thioacyl intermediate at δ = 195.9 ppm in the presence of papain under the cryoenzymological conditions of -6°C in 25 percent aqueous dimethyl sulfoxide (*48*) (Fig. 4). Moreover, the thioacyl species is clearly a productive intermediate since the decrease in its signal intensity was accompanied by an increase in the product resonance and by release of free enzyme (half-life, $\approx$96 minutes) determined by titration of its thiol group. The line width of the resonance at 195.9 ppm was 25 $\pm$ 5 Hz.

Similar experiments with chymotrypsin and the nonspecific substrate [^{13}C=O]*p*-nitrophenylacetate (δ = 170.4 ppm) have allowed observation of an acyl adduct (δ = 174.0 ppm) by ^{13}C NMR spectroscopy (*49*).

The trypsin-catalyzed hydrolysis (Fig. 5) of the highly specific substrate N^{α}-carbobenzyloxy-L-lysine-*p*-nitrophenylester (Z-lys-pNP) has been studied in detail under cryoenzymological conditions by both spectrophotometry (*50*) and ^{13}C NMR spectroscopy (*51*). The kinetic data from both techniques (*50, 51*) confirm that the kinetics and mechanism under cryoenzymological conditions are essentially the same as those determined at ambient temperatures by rapid reaction techniques (*52*). The hydrolysis of [1-^{13}C]Z-lys-pNP (**S** in Fig. 5A, δ = 173.6 ppm) by trypsin was monitored by the decrease in intensity of this signal and the increase in the signal arising from the product ($\mathbf{P_2}$ in Fig. 5A, δ = 177.7 ppm) at -21°C in 41 percent aqueous dimethyl sulfoxide (Fig. 5). The continued formation of product ($\mathbf{P_2}$) after all the substrate has been consumed (Fig. 5B) provides indirect evidence for an enzyme-bound intermediate whose line width is much greater than that of the free substrate or product and is therefore not directly observable in the individual ^{13}C NMR spectra of Fig. 5. Improvement of the signal-to-noise ratio by summation of sets of the individual spectra in Fig. 5 made it possible to use difference techniques and allowed direct observation (Fig. 6B) at -21°C of a resonance (δ = 176.5 ppm) that was assignable to the acyl enzyme on the basis of its chemical shift and the kinetics of its breakdown-formation. Experiments at -1.5°C (Fig. 6A) showed that the acyl intermediate was much more readily detected at this higher temperature, the resonance being clearly resolved in individual spectra as a result of the smaller line width of the resonance at -1.5°C compared to that at -21°C (22 $\pm$ 3 Hz and 100 $\pm$ 10 Hz, respectively). This illustrates one of the main difficulties of a combined NMR–cryoenzymological ap-

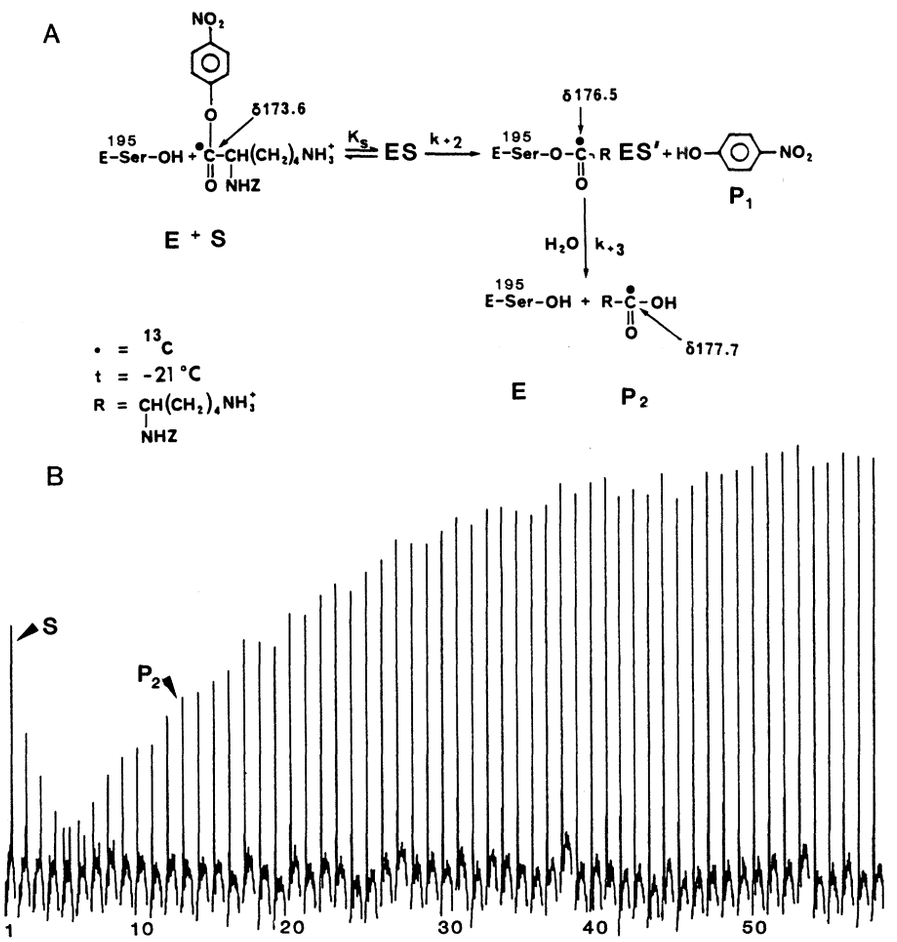


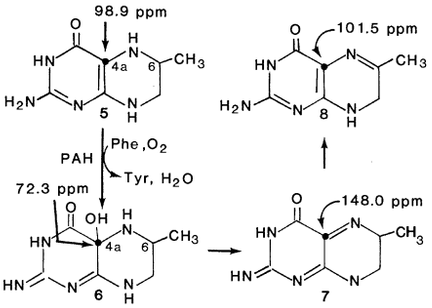
Fig. 5. (A) Reaction of [1-^{13}C]Z-lys-pNP, 0.83 m*M*; active trypsin, 0.7 m*M*; 1 m*M* HCl (apparent *p*H, 3.2); and 40 percent (by volume) dimethyl sulfoxide (sample volume, 10 ml; sample temperature, -21°C); NHZ, carbobenzloxy-. The reaction was initiated by the addition of [1-^{13}C]Z-lys-pNP; after mixing ($\approx$1 minute) NMR data acquisition (B) commenced within 3 minutes. Each spectrum (1 to 58) represents 10,000 accumulations (time per spectrum, 41 minutes) recorded sequentially (*51*). The spectral width was 0 to 220 ppm, but only the region from 172 to 180 is shown. [Reprinted with permission from the *Biochemical Journal*]

proach in which the line width of enzyme-bound species increases dramatically as the cryosolvent viscosity increases on lowering the temperature.

Coenzyme Enrichment: ^{13}C-Enriched Coenzymes and Cofactors

An early and cogent example is provided by the experiments of Ghisla *et al.* (*53*), using flavin mononucleotide enriched in ^{13}C at the 4a position. On addition to bacterial luciferase in 20 percent aqueous ethylene glycol and reduction with dithionite, two signals were observed, one at 103 ppm arising from bound reduced flavin mononucleotide and one at 104 ppm for the free reduced flavin. On cooling to −15°C, molecular oxygen was added, whereupon the spectrum showed a sharp signal at 137 ppm due to reoxidized flavin and a broad signal, at least 50 Hz wide, arising from the 4a oxygen adduct of the coenzyme-enzyme complex.

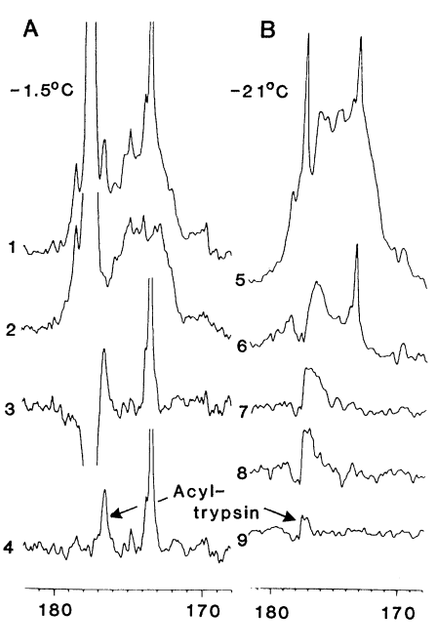
Recently an elegant ^{13}C NMR study (*54*) on the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) by phenylalanine hydroxylase (PAH), an enzyme that requires a pteridine cofactor, has been described. The reduced cofactor, in the tetrahydro form, combines with molecular oxygen to form an active hydroxylating species. Unlike the tightly bound flavin coenzymes (see above), the pterin cofactor dissociates from the enzyme at the end of each catalytic cycle and is subsequently regenerated, an event that alleviates, to some extent, the problems that arise from the large line widths of tightly bound enzyme intermediates.



Scheme 3 (*54*). [Reprinted with permission from the *Journal of the American Chemical Society*]

In the event, 6-methyltetrahydropterin (**5** in Scheme 3) was synthesized with C-4a enriched to 90 atom percent and added to an activated PAH solution (at 0°C) saturated with oxygen. It was necessary to perform the initial intermediate of the reaction at 0°C because of its instability at higher temperatures (half-life = 2.0 minutes at 23.2°C). After 3.5 minutes at 0°C, methanol was added to give a 40 percent aqueous methanol solution while cooling to −30°C. Figure 7 shows the ^{13}C NMR spectra of the tetrahydropterin and of subsequent reaction products after the addition of oxygen and dehydration (Scheme 3). It was concluded that C-4a was hydroxylated (**5** → **6** in Scheme 3) to give a resonance at 72.3 ppm. Dehydration of **6** gave **7** with a C-4a resonance at 148.0 ppm. Other structural possibilities such as ring-opened oxenoid species were ruled out since the spectra depicted in Fig. 7 were also accumulated with ^{1}H coupling. The continuous observation of coupling constants between C-4a and H-6 proved that the pterin ring system remained intact.

Another coenzyme, pyridoxal phosphate, is a cofactor in a wide variety of enzyme-mediated reactions (*55, 56*). Researchers have used ^{13}C NMR to examine the binding of ^{13}C-enriched pyridoxal phosphate to D-serine dehydratase (molecular weight 46,000) and L-glutamate decarboxylase (subunit molecular weight 50,000) (*57*). In aqueous solution [4′,5′-$^{13}C_2$]pyridoxal phosphate shows two sharp resonances at δ = 196.8 and 88.4 ppm (for the 4′-aldehyde carbonyl and its hydrate, respectively) and one at δ = 62.8 ppm (for the 5′-methylene carbon). On binding to D-serine dehydratase, two resonances not found in the native enzyme are observed at 167.7 and 62.7 ppm with line widths of 24 ± 3 and 48 ± 3 Hz, respectively. The former resonance has been assigned to a Schiff's base formed between the 4′-^{13}C aldehyde group of pyridoxal phosphate and an ε-amino function of a lysine residue of the enzyme. The 5′-methylene carbon remains virtually unaltered save for line-broadening. The twofold increase in line width for the carbon-5′ signal compared with the carbon-4′ signal is to be expected for methylene and methine carbons. The τ_r of the enzyme calculated from both these line width values was 16 ± 2 nsec. This is significantly less (see above) than expected for a ^{13}C nucleus rigidly bound to a protein of molecular weight 46,000 and suggests that the cofactor has some degree of motional freedom.



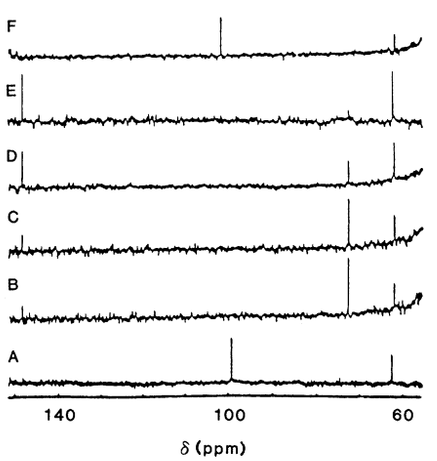


Fig. 6 (left). The ^{13}C NMR spectra of the reaction of (A) [1-^{13}C]Z-lys-pNP, 1.8 m*M*; active trypsin, 0.64 m*M*; 1 m*M* HCl (apparent *p*H, 3.02); and 40 percent (by volume) dimethyl sulfoxide (sample volume, 10 ml; sample temperature, −1.5°C). The reaction was initiated as described in Fig. 5, and spectra 1 to 15 (not shown) were recorded sequentially in the manner of Fig. 5B. Each spectrum resulted from 5000 accumulations (time per spectrum, 20.5 minutes). A 10-Hz exponential weighting factor was used. Spectra 1 and 2 represent the sums (25,000 accumulations of spectra 1 to 5 and spectra 11 to 15, respectively). Spectrum 3 is the difference between spectra 1 and 2. Spectrum 4 is spectrum 1 from which natural-abundance trypsin and product have been subtracted. (B) The free induction decay spectra from which the spectra in Fig. 5B were obtained were added together in groups of ten. Spectrum 5 represents the sum of spectra 2 to 11 (of Fig. 5B). Spectra 6 to 9 represent progressive subtractions of added spectra 12 to 21, 22 to 31, 32 to 41, and 49 to 58 from which product and natural-abundance trypsin resonances had been subtracted. Fig. 7 (right). The ^{13}C NMR spectra of the 4a-^{13}C intermediates described in the text and Scheme 3 (*54*) at −30°C (the signal at 61.5 ppm is due to 20 m*M* tris buffer): (A) [4a-^{13}C]6-methyltetrahydropterin (structure **5** in Scheme 3) prior to addition of PAH and phenylalanine; (B) spectrum acquired after mixing with PAH, time = 19 to 54 minutes (100 scans); (C) time = 64 to 170 minutes (260 scans); (D) time = 228 to 258 minutes (200 scans); (E) time = 360 to 400 minutes (100 scans); (F) solution after 580 minutes, allowed to warm to 30°C for 3 hours (300 scans). [Reprinted with permission from the *Journal of the American Chemical Society*]

The failure to detect any ^{13}C pyridoxal phosphate resonances on binding with L-glutamate decarboxylase presumably results from the fact that this enzyme is a hexamer (effective molecular weight 300,000). In this case, the bound pyridoxal phosphate does not have any detectable librational freedom.

Conclusions and Prognosis

From the discussion presented here it is clear that the stage is now set for intensive research into the mechanism of enzyme action with cryoenzymology. Although most of the enzymes discussed above lie in the molecular weight range of 20,000 to 35,000, an upper figure of ~50,000 probably represents the maximum convenient size for ^{13}C NMR studies on currently available superconducting instruments with substrates and inhibitors. The use of ^{13}C-enriched coenzymes has added another powerful tool which not only broadens the range of accessible enzymes but also appears to be independent of enzyme molecular weight, especially in those cases where free coenzyme can be observed. For example, the use of ^{13}C-enriched pyridine nucleotides, which has been restricted in the past to the in vivo determination of intracellular coenzyme pools and their redox status (*58*), can be extended to a wide range of enzymes that use nicotinamide adenine dinucleotide and its reduced form as cofactors.

Finally, we can expect exciting developments in the application of ^{13}C solid-state NMR spectroscopy to structural problems in enzyme complexes, a field which, although still in its infancy, already shows considerable promise (*59*).

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