geometry the exciton model predicts a change in kinetics of triplet sublevel population and decay as well.[7] This change should register as a change in optimum phase-angle setting of the lock-in amplifier tuned to the light modulation frequency. In accordance with this prediction, we find that the optimum phase-angle settings for recording of the two triplets differ by about 90°.

Numerous techniques have been used in investigations of the structure and properties of chlorophyll aggregates involved in photosynthesis.[6,7] The evaluation of the data in part must rely on a data base provided by studies of model systems. It appears that H_2(TPPS) could be an ideal model system for the exploration of the effects of dimerization on physical and chemical properties of porphyrin-like structures.

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Registry No. Na^+, 17341-25-2; K^+, 24203-36-9; K^+ 18-crown-6, 31270-13-0; tetra(4-sulfonatophenyl)porphyrin, 39174-47-5.

Structure and Stereochemistry of Tetrahedral Inhibitor Complexes of Papain by Direct NMR Observation

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Application of ^{13}C NMR spectroscopy under cryoenzymological conditions to the study of the thiol protease papain has revealed structural evidence[4] for the acylenzyme (ES′) in the overall mechanism of eq 1. While it is generally assumed that a tetrahedral intermediate is formed during the acylation and deacylation steps leading from the Michaelis complex (ES) to the products [P_1 (= amine or alcohol) and P_2 (= carboxylic acid)] and the enzyme (E), the evidence for such a labile intermediate is still indirect,[5] although a spectrophotometric study has indicated that a tetrahedral intermediate can be observed at subzero temperature[6] with papain.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

Ketonic inhibitors whose functionality mimics the scissile peptide bond have also provided useful models for binding in serine proteases where it has been demonstrated that stabilized covalent tetrahedral species can be characterized by ^{13}C NMR.[7,8] So far, no *peptide* inhibitor has shown evidence of a covalent, tetracoordinated species,[9] nor have productive tetrahedral complexes been observed with thiol proteases by NMR methods.

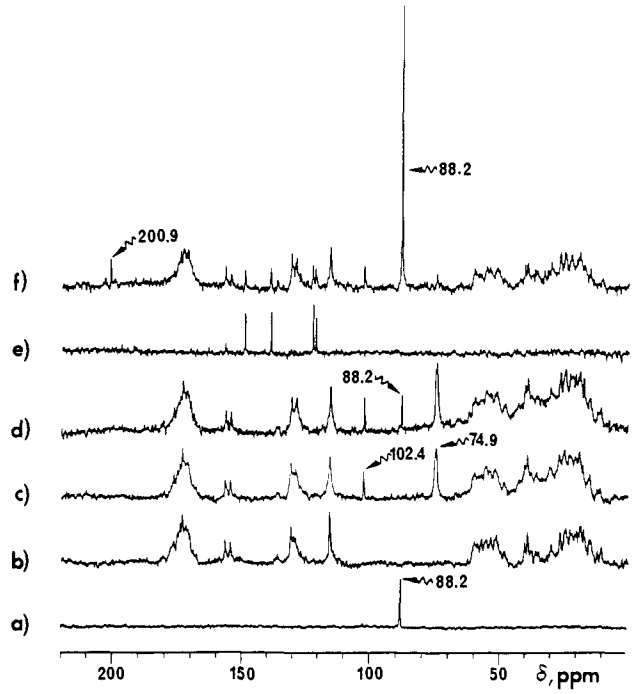
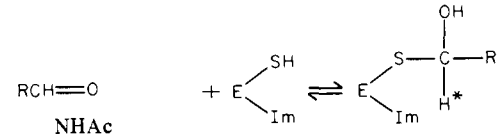


Figure 1. Spectra: 75.47 MHz (^{13}C), proton decoupled at 4–5 W with decoupler power reduced to 0.4 W for 0.2–0.4 s after each acquisition time of 0.2 s, 10-Hz exponential weighting and 8 K time domain data points, 10-μs pulse width (35 μs = 90° pulse). Chemical shifts are relative to Me_4Si. *N*-Acetylphenylalanyl[1-^{13}C]glycinal concentrations; fully active papain concentrations; D_2O, % (v/v); pH; no. of accumulations (all in 10 mM sodium phosphate): (a) 3.41 mM; 0.00 mM; 80; 7.0; 1030. (b) 0.00 mM; 0.61 mM; 26; 7.2; 29 000. (c) 0.69 mM; 0.72 mM; 26; 7.1; 29 000. (d) 1.71 mM; 0.62 mM, 33; 7.1; 32 000. (e) 0.00 mM; 0.00 mM; 33; 7.2; 44 000 plus 2,2-dipyridyl disulfide 1.37 mM. (f) As (d) except pH 4.1 plus 2,2′-dipyridyl disulfide 1.5 mM (see legend Figure 2).

In order to provide the necessary spectroscopic data for the eventual characterization of a tetrahedral adduct of papain, (*N*-acetylphenylalanyl)glycinal (**1**) was selected on the basis of its



1, R = $PhCH_2CHCONHCH_2$
3, R = $PhCONHCH_2$

potent inhibitory properties and the suggestion[10] that hemithioacetal (tetrahedral) structures (as **2**) were formed by addition of the cys-25 thiolate of papain to the aldehyde carbonyl of peptide inhibitors. Using *N*-benzoylamino[1-^{13}C]acetaldehyde (**3**) and papain, Lowe[11] proved, by an ingenious cross-saturation ^1H-NMR experiment, that the magnetization transfer data were in full accord with the presence of a proton (H*) attached to a tetrahedral carbon (τ(H*) 3.81; $^1J(^1H-^{13}C)$ = 183 Hz) although direct observation of a hemithioacetal inhibitor complex (**2**) per se was not possible in this experiment. It was also shown that the aldehyde and not the hydrated form was the true inhibitor of papain.[11] The wide range and diagnostic power of ^{13}C NMR suggested that a complete structural assignment could be made for an aldehyde inhibitor–papain complex, and we now report on the results of such an experiment.

Reaction of [1-^{13}C]aminoacetaldehyde dimethyl acetal (prepared from [1-^{13}C]glycine (90% ^{13}C atom percent)) with *N*-

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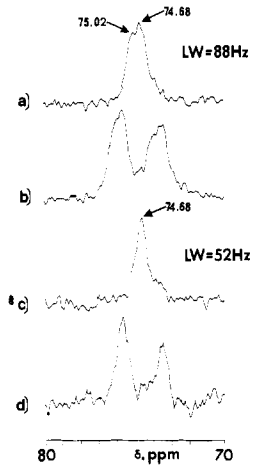


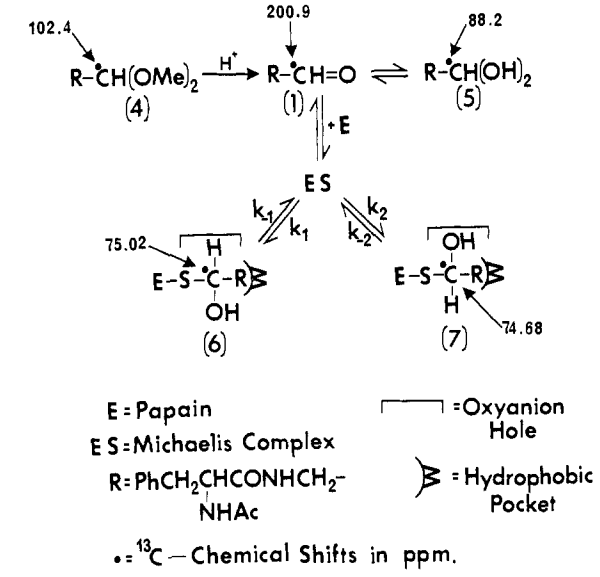
Figure 2. Spectra a and c are proton-decoupled and b and d nondecoupled expansions recorded under the conditions of Figure 1d (32 000 and 128 000 accumulations, respectively). Spectra c and d were recorded 1.5 h after addition of 2,2′-dipyridyl disulfide (5 mg) to the same sample (Figure 1d). At the end of the experiment, 0.4 M HCl (0.06 mL) was added (→pH 4.1) and spectrum 1f (Figure 1) recorded.

acetylphenylalanine gave *N*-acetyl-L-phenylalanyl[1-^{13}C]glycinal dimethyl acetal (**4**), which was hydrolyzed directly before use. The ^{13}C NMR spectrum of the glycinal peptide (Figure 1a) reveals that a substantial amount (95%) of hydrate **5** (δ(C) 88.2) is present in D_2O (80%) together with small signals (not shown in 1000 accumulations) for aldehyde **1** (200.9 ppm) and unhydrolyzed acetal *4* (102.4 ppm). Addition of the glycinal **1** to papain (Figure 1b) at 25 °C resulted in the disappearance of the hydrate resonance at 88.2 ppm and the appearance of a new signal at 74.9 ppm (Figure 1c). The latter resonance is assigned to the hemithioacetal structure **2** on the basis of its close correspondence of chemical shift to the asymmetric carbon of a model hemithioacetal prepared from acetaldehyde and 1-mercaptoethanol (δ(C-1) 73.27). Addition of a 3-fold excess of the glycinal (**1**) to the solution resulted in enhancement of the hemithioacetal signal at 74.9 ppm and reappearance of the hydrate **5** at 88.2 ppm (LW = 14 Hz) (Figure 1d), which, on the basis of line-width (LW) measurements, is clearly not in *rapid* chemical exchange ($K_e << 6 \times 10^3$ s^{-1}) with the hemithioacetal (LW = 88 Hz).

Addition of 2,2′-dipyridyl disulfide (DDS) (spectrum, Figure 1e), a site-directed reagent for the thiolate ion (cys-25), at pH 4.1 leads to the disappearance of the hemithioacetal signal at 74.9 ppm with concomitant increase of the signal for the hydrate (**5**) at 88.2 ppm and free glycinal **1** (200.9 ppm) (Figure 1f) confirming that papain and the hemithioacetal **2** are in equilibrium. Papain (90% activity) was recovered from the latter experiment.

Careful examination of the signal at 74.9 ppm by scale expansion reveals that this resonance is a composite of *two broad signals* (total LW = 88 Hz) *centered at 74.68 and 75.02 ppm* (Figure 2a). Incubation of the complex with DDS at pH 7.0 preferentially removes the resonance at 75.02 ppm (Figure 2c) with concomitant increase in concentration of hydrate **5** (not shown). Since the signal at 74.68 ppm decreases on adjusting the pH to 4.1, it is clear that *two* diastereomeric hemithioacetals (**6** and **7**, Scheme I) have been formed, which can be titrated separately, one of which is more stable than the other at pH 7. This differentiation also allowed the ^{13}C–H coupling constants for the resonances at 75.02 and 74.68 ppm to be assigned the values of 155 and 160 Hz, respectively[12] (Figure 2b,d). A model in which **1** is placed within the binding site at the S_1 subsite and the newly formed tetrahedral carbon aligned with the oxyanion hole (see Scheme I) in the "productive" mode[13] of binding leads

Scheme I



to the suggestion that formation and breakdown of the neutral "nonproductive" hemithioacetal **6** are facilitated by general acid catalysis and general base catalysis, respectively. Since equal amounts of both diastereoisomers are formed, the equilibrium constant for the formation/breakdown of each must be approximately equal. The diastereoisomer in the "productive" binding mode (**7**) has its hemithioacetal OH in the oxyanion hole, and its formation/breakdown is not subject to general acid/base catalysis by histidine-159. According to Scheme I, k_1/k_{-1} equals k_2/k_{-2}, but $k_1 >>> k_2$ and $k_{-1} >>> k_{-2}$,[14] leading to the tentative assignments **6** and **7** to the "nonproductive" (δ 75.02) and "productive" (δ 74.68) structures, respectively.

In summary, the observation of both diastereoisomers of the hemithioacetals **6** and **7** formed by the complexation of papain and the inhibitory aldehyde *N*-acetylphenylalanylglycinal and of their differentiation via NMR titration with DDS at pH 7 and 4 has provided both a valuable three-dimensional probe of the active-site stereospecificity of papain in solution and an excellent set of parameters whereby productive tetrahedral intermediates can eventually be recognized. It is also of interest that in the case of tetrahedral ketonic inhibitor adducts of trypsin[8] and pepsin,[7] only *one* diastereomeric form was observed, indicating a greater stereospecificity for these inhibitors at the active site. The stereospecificity of aldehyde binding in serine proteases[16] is now under investigation using similar ^{13}C NMR methods.

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Registry No. 1, 86921-93-9; **4**, 86921-94-0; [1-^{13}C]aminoacetaldehyde dimethyl acetal, 68921-95-1; *N*-acetyl-L-phenylalanine, 2018-61-3.

(12) These values can be compared with the results of cross-saturation ^{1}H NMR studies on the complex *N*-benzoylamino[1-^{13}C]acetaldehyde with papain for which J = 173 Hz was estimated.[11] It should be noted that using the cross-saturation method it would not be possible to resolve the two diastereoisomers.

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(14) The diastereoisomer (δ 75.02)[15] binding in the nonproductive mode[13] is in rapid equilibrium with the Michaelis complex (ES) while the diastereoisomer (δ 74.68)[15] is in the productive mode and in slow equilibrium with the Michaelis complex (ES). Thus at pH 7 the diastereoisomer (δ 75.02) binding in the nonproductive mode will be in rapid equilibrium with the free enzyme and will be titrated by DDS. At low pH the rate of dissociation of *N*-acetylphenylalanylglycinal increases[13] and the isomer **7** (δ 74.68) is titrated.

(15) The position and separation of chemical shift in the spectrum of isomers **6** and **7** is in accord with the diastereomeric hemithioacetals prepared from **1** and L-cysteine, which display signals at 75.8 and 76.8 ppm.

(16) See, e.g.: James, M. N. G.; Sielecki, A. R.; Braeyer, G. D.; Delbaere, L. J. T.; Bauer, C.-A. *J. Mol. Biol.* **1980**, *144*, 43.