Lakowicz, J. R., & Cherek, H. (1981a) *J. Biochem. Biophys. Methods 5*, 19–35.
Lakowicz, J. R., & Cherek, H. (1981b) *J. Biol. Chem. 256*, 6348–6353.
Laws, W. R., & Shore, J. D. (1978) *J. Biol. Chem. 253*, 8593–8597.
Laws, W. R., & Brand, L. (1979) *J. Phys. Chem. 83*, 795–802.
Meech, S. R., O'Connor, D. V., Roberts, A. J., & Phillips, D. (1981) *Photochem. Photobiol. 33*, 159–172.
Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369–4377.
Vallee, B. L., & Hoch, F. L. (1957) *J. Biol. Chem. 225*, 185–189.
Ware, W. R., Lee, S. K., & Chow, P. (1968) *Chem. Phys. Lett. 2*, 356–358.
Ware, W. R., Lee, S. K., Brant, G. J., & Chow, P. P. (1971) *J. Chem. Phys. 54*, 4729–4737.
Weber, G. (1961) *Nature (London) 190*, 27–29.

Proton and Fluorine Nuclear Magnetic Resonance Spectroscopic Observation of Hemiacetal Formation between *N*-Acyl-*p*-fluorophenylalaninals and α-Chymotrypsin†

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ABSTRACT: Proton nuclear magnetic resonance (NMR) spectroscopy shows that the free aldehyde and not the hydrate of *N*-acetyl-DL-*p*-fluorophenylalaninal binds to α-chymotrypsin. A proton NMR cross-saturation experiment shows that the initial noncovalent complex is in equilibrium with a hemiacetal formed between the aldehyde and the active site serine residue. Fluorine NMR spectra of *N*-acetyl-DL- (and *N*-acetyl-L-) *p*-fluorophenylalaninal in the presence of α-chymotrypsin show separate signals for the hemiacetal complex, the bound aldehyde, the free aldehyde, and the free hydrate. *N*-Benzoyl-DL-*p*-fluorophenylalaninal fluorine NMR signals are also observed for all species except the bound aldehyde form in the presence of α-chymotrypsin. The D and L enantiomers of the hydrate of the *N*-acetyl aldehyde inhibitor give separate fluorine NMR signals, arising from chemical exchange between the L-aldehyde–α-chymotrypsin complex and the free L hydrate. The enzyme-bound inhibitor fluorine signals disappear upon proton decoupling due to a negative nuclear Overhauser effect. Upon gated decoupling of protons, the L hydrate and free aldehyde fluorine signals are reduced in intensity relative to that of the D hydrate signal in the racemate aldehyde complex. This is attributed to a saturation transfer of the heteronuclear nuclear Overhauser effect.

Peptide aldehydes related to substrates have proven to be potent inhibitors of serine proteases (Aoyagi et al., 1969; Kondo et al., 1969; Kawamura et al., 1969; Ito et al., 1972; Thompson, 1973, 1974). Thompson suggested that the tighter binding of the aldehydes derives from stabilization of a hemiacetal tetrahedral adduct formed between the enzyme active site serine and the aldehyde carbonyl. This hemiacetal complex is believed to resemble the transition-state structure in substrate hydrolysis. Enzymes are predicted to bind transition-state structures more tightly than ground-state structures (Pauling, 1946; Wolfenden, 1972; Lienhard, 1972, 1973), and hence, any "transition-state analogue" such as the aldehyde inhibitors of the serine proteases should have a higher affinity for the enzyme than substrate or product analogue structures. Evidence for support of the hemiacetal structure has been indirect, as for example, Lewis & Wolfenden's (1977) secondary deuterium isotope effect on the association of benzamidoacetaldehyde to the cysteine protease papain, which suggests formation of a thiol–hemiacetal.

Gorenstein et al. (1976) had, in fact, shown that simply binding of an aldehyde (cinnamaldehyde) to a serine protease (α-chymotrypsin) was not sufficient evidence of itself to warrant the conclusion of transition-state stabilization in its binding. This proton nuclear magnetic resonance (NMR) study of the cinnamaldehyde binding to α-chymotrypsin (Cht) gave no evidence for the putative hemiacetal–enzyme complex. Similarly Breaux & Bender (1975) in a UV–vis spectroscopic study of substituted cinnamaldehydes binding to Cht could find no evidence in support of the transition state analogue, hemiacetal structure. Lowe & Nurse (1977) introduced an NMR double resonance experiment that provided strong evidence in favor of the hemiacetal structure for the hydrocinnamaldehyde–Cht complex (as originally suggested by Schultz & Cheerva, 1975). In this double resonance experiment the aldehydic proton of the aldehyde in the presence of Cht was cross saturated on proton irradiation in the region expected for the hemiacetal formed with the active site serine. In confirmation of Gorenstein's et al. (1976) earlier conclusion on cinnamaldehyde, no cross-saturation effects were noted with this aldehyde and Cht (Lowe & Nurse, 1977).

In a collaborative experiment, Lowe, Schultz, Gorenstein, and co-workers (Chen et al., 1979) applied proton NMR spectroscopy to the interaction of *N*-benzoyl- and *N*-acetyl-L-phenylalaninals with Cht and dehydroalanine-195–α-chymotrypsin. From line-width changes and cross-saturation effects it was shown that these specific aldehyde transition-state analogues do bind as the hemiacetal to Cht. Proton NMR signals for the hemiacetal structure, however, were never directly observed and were only inferred from the selective

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cross-saturation experiments [see also Wyeth et al. (1980)].

We now present a proton and fluorine NMR investigation of *N*-acetyl- and *N*-benzoyl-DL-*p*-fluorophenylalaninals and *N*-acetyl-L-*p*-fluorophenylalaninal binding to Cht, which provides the first direct observation of signals (fluorine) representing the hemiacetal structure.

Experimental Procedures

Materials

α-Chymotrypsin (bovine pancreatic) was purchased from Sigma Chemical Co. as a 3 times crystallized and lyophilized, salt-free type II powder. Active site titration of the enzyme followed the method of Schonbaum et al. (1961) and routinely yielded 80–85% active sites.

DL-*p-Fluorophenylalanine methyl ester hydrochloride* was prepared and purified by the method previously described, mp 179–181 °C (Gammon et al., 1972).

*N-Acetyl-*DL-*p-fluorophenylalanine Methyl Ester.* To a saturated solution of sodium bicarbonate (150 mL) was added DL-*p*-fluorophenylalanine methyl ester hydrochloride (4.8 g, 20.5 mmol) and ethyl acetate (150 mL). The ice-cooled mixture was shaken vigorously, and the phases were separated. The aqueous phase was extracted twice with ethyl acetate (2 × 40 mL). The ethyl acetate fractions were combined, dried over anhydrous sodium sulfate, and filtered, and the solvent was removed under vacuum on a rotary evaporator leaving a gummy residue. This was dissolved in anhydrous ethyl acetate (100 mL), and acetic anhydride (3.6 mL, 38.2 mmol) was added dropwise with stirring over 15 min. The solution was stirred at room temperature for 90 min further. The ethyl acetate solution was extracted with 10% aqueous sodium bicarbonate (150 mL), dried over anhydrous sodium sulfate, and filtered, and the solvent was removed under vacuum leaving a gummy residue. This was dissolved in anhydrous ethyl ether (10 mL) and diluted with *n*-hexane (60 mL), yielding a white product (3.6 g, 73.5%): mp 64–65 °C (from ethyl ether–*n*-hexane); IR (KBr) ν_{max} 1625 (CONH), 1745 ($COOCH_3$), and 3315 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.85–7.15 (m, 4 H, aromatic), 6.1–6.55 (br, 1 H, NH), 4.62–5.0 (m, 1 H, CH), 3.8 (s, 3 H, ester), 3.0–3.2 (d, 2 H, CH_2), and 2.1 (s, 3 H, $NHCOCH_3$). Anal. Calcd for $C_{12}H_{14}O_3NF$: C, 60.25; H, 5.86; N, 5.86; F, 7.95. Found: C, 59.95; H, 5.82; N, 5.81; F, 8.17.

*N-Acetyl-*DL-*p-fluorophenylalaninal* was synthesized by the general method of Ito et al. (1975). *N*-Acetyl-DL-*p*-fluorophenylalanine methyl ester (1.2 g, 5.02 mmol) was dissolved in dry dimethoxyethane (35 mL) and cooled to about −60 °C. A solution of diisobutylaluminum hydride (7.0 mL, 25% in toluene) was added over 30 min with stirring, and the reaction mixture was stirred at −60 to −65 °C for a further 2 h. HCL (2 N, 55 mL) was added slowly to the reaction mixture, which was then allowed to warm to −5 °C. The reaction mixture was extracted first with ethyl acetate (2 × 30 mL) and then with chloroform (5 × 30 mL). Ethyl acetate extracts were combined and washed with saturated NaCl solution, dried (Na_2CO_3), and evaporated in vacuo to yield an oil, which was mainly unreacted starting material. The chloroform extracts were washed with saturated NaCl solution and dried ($MgSO_4$), and the solvent was removed in vacuo to give a white solid (0.41 g, 39.1%): mp 94–96 °C (recrystallized from ethyl acetate–*n*-hexane as fine white needles); IR (KBr) ν_{max} 1630 (CONH), 1720 (CHO), and 3310 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.5 (s, 1 H, CHO), 6.8–7.25 (m, 4 H, aromatic), 5.75–6.2 (br, 1 H, NH), 4.4–4.8 (m, 1 H, CH), 2.95–3.15 (d, 2 H, CH_2), and 1.96 (s, 3 H, $NHCOCH_3$); mass spectrum molecular ion at *m/e* 209. Anal. Calcd for $C_{11}H_{12}O_2NF$: C, 63.16; H, 5.74; N, 6.70; F, 9.10. Found: C, 63.00; H, 5.75; N, 6.96; F, 9.27.

L-*p-Fluorophenylalanine methyl ester hydrochloride* was prepared and purified by the method described by Gammon et al. (1972) for the preparation of DL-*p*-fluorophenylalanine methyl ester hydrochloride. Crystallization from methanol–ether gave colorless needles (yield 94% on the basis of L-*p*-fluorophenylalanine): mp 203 °C; ^1H NMR (D_2O) δ 6.85–7.1 (m, 4 H, aromatic), 4.0–4.3 (m, 1 H, CH), 3.6 (s, 3 H, ester), and 3.0–3.15 (d, 2 H, CH_2). Anal. Calcd for $C_{10}H_{13}O_2NFCl$: C, 51.39; H, 5.57; N, 6.00. Found: C, 51.33; H, 5.53; N, 6.02.

*N-Acetyl-*L-*p-fluorophenylalanine methyl ester* was prepared by the method as described for the preparation of *N*-acetyl-DL-*p*-fluorophenylalanine methyl ester. Crystallization from anhydrous ether–hexane gave fine needles (yield 74.0%): mp 91–91.5 °C; IR (KBr) ν_{max} 1620 (CONH), 1745 (C=O-CH_3), and 3320 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.96–7.16 (m, 4 H, aromatic), 5.90–6.15 (br, 1 H, NH), 4.75–4.96 (m, 1 H, CH), 3.72 (s, 3 H, ester), 2.92–3.15 (d, 2 H, CH_2), and 1.96 (s, 3 H, $NHCOCH_3$); mass spectrum molecular ion at *m/e* 239. Anal. Calcd for $C_{12}H_{14}O_3NF$: C, 60.25; H, 5,86; N, 5.86; F, 7.95. Found: C, 60.13; H, 5.92; N, 5.82; F, 7.92.

*N-Acetyl-*L-*p-fluorophenylalaninal* was prepared by the method as described for the preparation of *N*-acetyl-DL-*p*-fluorophenylalaninal. Crystallization from ethyl acetate–isopropyl ether gave fine colorless needles (yield 42.0%): mp 105–107 °C; IR (KBr) ν_{max} 1625 (CONH), 1725 (C=O), 3300 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.6 (s, 1 H, CHO), 6.95–7.30 (m, 4 H, aromatic), 6.35–6.7 (br, 1 H, NH), 4.4–4.85 (m, 1 H, CH), 2.95–3.15 (d, 2 H, CH_2), and 1.95 (s, 3 H, $NHCOCH_3$); mass spectrum molecular ion at *m/e* 209. Anal. Calcd for $C_{11}H_{12}O_2NF$: C, 63.16; H, 5.74; N, 6.70; F, 9.10. Found: C, 62.98; H, 5.79; N, 6.55; F, 9.22.

*N-Benzoyl-*DL-*p-fluorophenylalanine.* To the cooled clear solution of DL-*p*-fluorophenylalanine (1.83 g, 10 mmol) in distilled water (18 mL) was added a saturated solution of sodium hydroxide (1.1 mL) and then freshly distilled benzoyl chloride (1.54 g, 11.0 mmol) over 30 min. The temperature was maintained at 0–5 °C during the course of benzoylation. The solution was kept alkaline by the simultaneous addition of 4% (w/v) sodium hydroxide. Then after the reaction mixture was stirred at the same temperature for a further hour, the cooled solution was just acidified with dilute hydrochloric acid. The white precipitate was filtered and washed with isopropyl ether (25 mL) and crystallized from aqueous methanol to yield 2.7 g (95%) of small, colorless needles: mp 164–166 °C; IR (KBr) ν_{max} 1630 (CONH), 1725 (C=O), and 3320 (NH) cm^{-1}; ^1H NMR (CD_3CN) δ 6.85–7.85 (m, 9 H, aromatic), 6.35–6.65 (br, 1 H, NH), 4.5–4.95 (m, 1 H, CH), and 2.8–3.0 (d, 2 H, CH_2). Anal. Calcd for $C_{16}H_{14}O_3NF$: C, 66.90; H, 4.88; N, 4.88; F, 6.62. Found: C, 66.97; H, 4.95; N, 4.91; F, 6.64.

*N-Benzoyl-*DL-*p-fluorophenylalaninol.* A solution of *N*-benzoyl-DL-*p*-fluorophenylalanine (2.87 g, 10 mmol) in dry tetrahydofuran (20 mL) was added to a slurry of $LiAlH_4$ (0.44 g) in dry ether (70 mL) with slow stirring over 30 min under a nitrogen atmosphere. This was refluxed for 5 h and left overnight with stirring at room temperature. The reaction mixture was kept under a nitrogen atmosphere throughout the reaction period. Water (2.0 mL) was added, the precipitate was filtered off, and the residue was extracted with hot ether (3 × 100 mL). The organic layers were combined and washed with 1% NaOH solution (2 × 150 mL). The organic layer was dried over Na_2SO_4, and evaporation of the solvent left a

white residue, which was extracted with hot petroleum ether (bp 35–60 °C; 3 × 150 mL) and filtered. This on cooling deposited mainly the side product *N*-benzyl-DL-*p*-fluorophenylalaninol: mp 80–82 °C (recrystallized from ethanol; yield 0.3 g, 11.6%); IR (KBr) λ_{max} 3260 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.90–7.35 (m, 9 H, aromatic), 4.0–4.4 (m, 1 H, CH), 3.8 (s, 2 H, $PhCH_2NH$), 3.5–3.65 (d, 2 H, CH_2OH), and 2.85–3.05 (d, 2 H, $FPhCH_2$). Anal. Calcd for $C_{16}H_{18}ONF$: C, 74.13; H, 6.94; N, 5.40; F, 7.30. Found: C, 73.96; H, 6.96; N, 5.32; F, 7.06.

The insoluble product in petroleum ether (bp 35–60 °C) was purified on a silica gel column with a mixture of benzene and petroleum ether, bp 35–60 °C (80:20), as the eluent. Recrystallization from benzene yielded pure *N*-benzoyl-DL-*p*-fluorophenylalaninol: mp 135–137 °C (yield 1.17 g, 62.2%); IR 1660 (CONH) and 3280 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.9–7.70 (m, 9 H, aromatic), 6.35–6.8 (br, 1 H, NH), 4.0–4.45 (m, 1 H, CH), 3.6–3.8 (d, 2 H, CH_2OH), and 2.85–3.05 (d, 2 H, $FPhCH_2$); mass spectrum molecular ion at *m/e* 273. Anal. Calcd for $C_{16}H_{16}O_2NF$: C, 70.33; H, 5.86; N, 5.13; F, 6.96. Found: C, 70.26; H, 5.94; N, 5.11; F, 6.76.

N-Benzoyl-DL-p-fluorophenylalaninal. Oxidation of *N*-benzoyl-DL-*p*-fluorophenylalaninol was carried out by the procedure generally described by Thompson (1977). A solution of *N*-benzoyl-DL-*p*-fluorophenylalaninol (0.589 g, 2.1 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Sigma Chemical Co.; 2.0 g, 10 mmol) in fresh doubly distilled dimethyl sulfoxide (13.0 mL) was rapidly stirred and treated dropwise with 1 mL of 2 M anhydrous H_3PO_4 in dimethyl sulfoxide. After 4 h 80 mL of 0.1 M sodium phosphate buffer, pH 8.0, was added and the solution extracted with ethyl acetate (3 × 35 mL). The ethyl acetate layer was washed with saturated aqueous $NaHCO_3$ (30 mL) and saturated saline (30 mL), dried over anhydrous Na_2SO_4, and filtered and the solvent removed under vacuum. Crude product was crystallized from benzene–hexane (yield 250 mg, 46%), mp 118–122 °C. This was further purified by preparative reverse-phase high-pressure liquid chromatography with a Whatman RSIL C_{18} column (Altec) and 45% aqueous acetonitrile as a mobile phase: IR (KBr) λ_{max} 1640 (CONH), 1730 (C=O), and 3300 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.75 (s, 1 H, HC=O), 6.95–7.85 (m, 9 H, aromatic), 6.55–6.85 (br, 1 H, NH), 4.8–5.1 (m, 1 H, CH), and 3.25–3.45 (d, 2 H, CH_2); mass spectrum molecular ion at *m/e* 271. Anal. Calcd for $C_{16}H_{14}O_2NF$: C, 70.84; H, 5.17; N, 5.17; F, 7.01. Found: C, 70.49; H, 5.28; N, 5.14; F, 7.23.

Methods

^1H and ^{19}F NMR spectra were recorded on a Bruker WP-80 spectrometer at 80 MHz (^1H) or ^1H NMR spectra on a 60-MHz Varian T-60 spectrometer. Chemical shifts in parts per million for ^1H NMR spectra are referenced to external Me_4Si and for ^{19}F NMR spectra are referenced to external CF_3CO-OH. Mass spectra were taken on an AEI MS-30 spectrometer. Infrared spectra were obtained on a Perkin-Elmer 727B spectrometer. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

Proton NMR Spectra. Cht was dissolved in D_2O and lyophilized several times before dissolving in 0.1 M phosphate buffer, in 99.9% D_2O containing 1 mM of ethylenediaminetetraacetic acid (EDTA), pD = 6.0. To this solution was added the aldehyde dissolved in dimethyl-d_6 sulfoxide so that the final concentration of dimethyl-d_6 sulfoxide was about 20% (v/v) and the aldehyde concentration was about 10–80 mM for *N*-acetyl-DL-*p*-fluorophenylalaninal (AcFPheal) or, in the ^{19}F NMR spectra, 10 mM for *N*-benzoyl-DL-*p*-fluorophenylalaninal (BzFPheal). Enzyme concentrations are corrected for active sites, while no correction for enzyme dimerization was made (Gammon et al., 1972). Cross-saturation experiments involved application of a 500-ms gated pulse of 0.1-V radio frequency intensity, immediately prior to the spectral sampling. The bandwidth of the F_2 was sufficiently narrow under these conditions to completely saturate the residual HOD for example without markedly saturating the signals of the aldehydes. Adequate spectra were obtained with 250–1000 scans in the quadrature detection mode at 25 °C.

Fluorine NMR Spectra. Samples were prepared as above but with 20% dimethyl sulfoxide. Normally spectra were obtained without broad-band proton decoupling, with 2000–8000 scans, 90° pulses, 0.5-s acquisition time, and 2.0-s relaxation delay. Gated-decoupled spectra had the broad-band proton irradiation gated on for a short period (5 ms–1 s) prior to and during the acquisition period (0.2 s) and gated off during a 2–10-s recovery period. All NMR solutions were freshly prepared from recrystallized aldehyde.

Determination of Inhibition Constants (K_i) to Cht. The inhibition constant was determined by standard steady-state kinetics (Dixon & Webb, 1964) against the substrate *N*-benzoyl-L-tyrosine ethyl ester. In a typical experiment, 100 μL of enzyme stock solution at pH 3 (0.01 mg/mL) was added to 2.9 mL of substrate solution in 50 mM phosphate buffer at pH 7.8 in a thermostated reaction cell (25 °C) of a Cary 210 or Varian 16 spectrometer, and the hydrolysis of substrate was followed by the linear increase of absorbance at 256 nm. A plot of $1/v_i$ against [I] for two or three different substrate concentrations (0.058–0.23 mM) gave straight lines. Reported inhibition constants and their standard deviations were calculated by a nonlinear least-squares fit of the data to the competitive inhibition, Michaelis–Menten equation (Dixon, 1953) with a PDP-11/03 minicomputer.

pD Measurements. Reported pD values represent corrected pH meter readings in D_2O, standardized against H_2O buffers; i.e., pD = "pH" + 0.41.

Hydration Constant for Aldehydes. The areas of the aldehyde and hydrate proton or fluorine signals were measured by utilizing the area measurement capability of the Bruker data system. Either ^1H or ^{19}F NMR spectra gave a hydration constant, K_H = [hydrate]/[aldehyde], of 11 ± 1 for a 20% Me_2SO–0.1 M phosphate, D_2O solution of the aldehydes.

Results and Discussion

As in our previous study with the unfluorinated aldehyde inhibitors (Chen et al., 1979), solubility limitations required that *N*-acetyl- and *N*-benzoyl-*p*-fluorophenylalaninals were added to the 20% dimethyl sulfoxide buffered enzyme solution. Hydration is much more rapid than the time taken to determine any of the NMR spectra. The pD for most experiments was 6.1–6.4. Kennedy & Schultz (1979) had previously shown that the equilibrium dissociation constant of *N*-benzoyl-L-phenylalaninal (BzPheal) to Cht varies by only a factor of 4 between pH 3 and 8, and thus pH effects on the fluorinated derivatives are expected to be small as well.

In most experiments the racemic mixture of the aldehydes was used, although only the L enantiomers are expected to bind especially tightly to the enzyme. Thus Kennedy & Schultz (1979) have shown that the D enantiomer of BzPheal has a K_i of 1.1 mM while the L enantiomer has a K_i of 0.035 mM (both in 12.5% Me_2SO). As shown in Table I, the inhibition constant of the L enantiomer of AcFPeal (0.79 mM) is ca. 0.5 the value of the DL racemate inhibition constant. This is nearly the same inhibition constant as the unfluorinated pure-L derivative (K_i = 0.7 ± 0.2 mM, Table I). All aldehydes behaved

Table I: Binding Dissociation Constants Found for *N*-Acyl-*p*-fluorophenylalaninals and Related Compounds to α-Chymotrypsin at pH 7.8

substrate analogue	K_i (mM)
DL-*N*-BzFPheal	0.062 ± 0.004[a]
DL-*N*-BzFPhe	>7[a]
L-*N*-BzPheal	0.036 ± 0.001[b]
L-*N*-BzPhe	27[c]
DL-*N*-AcFPheal	1.25 ± 0.27[a]
L-*N*-AcFPheal	0.79 ± 0.22[a]
L-*N*-AcPheal	0.7 ± 0.2[d]

[a] At 25 °C, 0.049 mM sodium phosphate, 0.095 M NaCl, and 20% Me_2SO. [b] From Kennedy & Schultz (1979); at 25 °C, 0.05 M sodium phosphate, 0.1 M NaCl, and 12.5% Me_2SO, pH 7.8. [c] From Bender & Kemp (1957). [d] From Chen et al. (1979); at 25 °C, 1 mM phosphate.

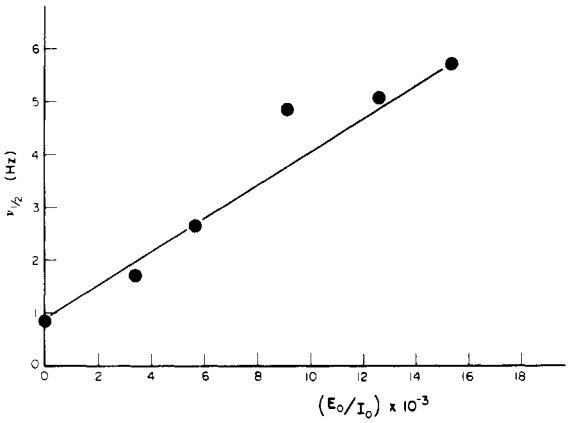


FIGURE 1: Plot of corrected line width of aldehyde proton resonance of DL-*N*-AcFPheal as a function of α-chymotrypsin/inhibitor ratio (E_0/I_0) at 25 °C, pD 6.0, and $[I]_0$ = 60 mM. $\nu_{1/2}$ is the line width (at half-height) of the aldehyde proton (●) plus the line width (at half-height) of the acetyl resonance in the absence of enzyme minus the line width (at half-height) of the acetyl resonance at the appropriate enzyme/inhibitor ratio.

as competitive inhibitors against *N*-benzoyl-L-tyrosine ethyl ester substrate.

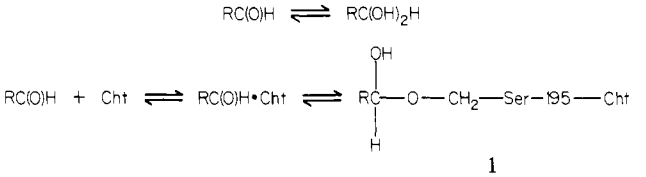
*N-Acetyl-*DL*-p-fluorophenylalaninal (AcFPheal) Proton NMR Spectra.* As was shown for the unfluorinated aldehyde (Chen et al., 1979), the proton NMR chemical shift of the aldehyde proton of DL-AcFPheal is unchanged upon addition of Cht while the line width, $\nu_{1/2}$, increases. A plot of the corrected line width of the CH proton of the aldehyde against the initial enzyme to inhibitor ratio (E_0/I_0) at pD 6.0 is shown in Figure 1. The line-width correction procedure described in Figure 1 eliminates any nonspecific line broadening due to increased viscosity of the solution upon enzyme addition. Only the aldehyde proton and not the hydrate proton broadens ($\nu_{1/2}$ is constant ±1 Hz, data not shown) and indicates that only the aldehyde binds to the enzyme. For a simple two-state equilibrium where $[I]_0 \gg [E]_0$ and thus $[EI] \sim [E]_0$ in all cases

$$\nu_{1/2} = \nu_{1/2,I} + \nu_{1/2,EI}([E]_0/[I]_0)$$

where $\nu_{1/2,I}$ and $\nu_{1/2,EI}$ are the line widths at half-height of the free and enzyme-bound inhibitor, respectively.

In Chen et al. (1979) it was established that a more complex binding scheme was required to explain additional proton NMR cross-saturation results. Following the double resonance experiment of Lowe & Nurse (1977) and Clark et al. (1977), we find that irradiation of DL-AcFPheal in the presence of Cht in the region expected for the hemiacetal formed with the active site serine residue (5.36 ppm) results in a 30% cross saturation of the aldehyde proton. The scheme for binding the unfluorinated aldehyde (and by inference our fluorinated aldehyde as well) should be modified to include subsequent formation of the covalently bound hemiacetal **1** from the initial noncovalent Michaelis complex:

Scheme I



1

In these proton NMR experiments evidence for the enzyme-bound forms of the aldehyde inhibitors has been provided only by indirect line-width and cross-saturation effects. Signals associated with the bound species were not observed. The broadening of the L-AcPheal and AcFPheal aldehyde signals with increasing enzyme suggests that the noncovalent aldehyde enzyme complex is in rapid chemical exchange with the free aldehyde on the NMR time scale. The free aldehyde, however, should be in slow exchange with the hemiacetal (Kennedy & Schultz, 1979; Chen et al., 1979), and thus while it should be feasible to observe a hemiacetal signal of the enzyme complex near 5.36 ppm, no signal has yet been identified. This is near the HDO signal, and a small, broad signal would not be easily identified.

Fluorine NMR Spectra. Fluorine-19 NMR spectroscopy does not suffer from this limitation, and separate enzyme–inhibitor complex signal(s) should be observable under slow chemical exchange conditions. Shown in Figures 2 and 3 are the ^{19}F NMR spectra of DL-AcFPheal in the absence and presence of Cht. In the proton-decoupled ^{19}F spectrum of this aldehyde (Figure 3a) two signals at −37.6 and −38.4 ppm (upfield from an external sample of trifluoroacetic acid standard, D_2O lock) are observed. The smaller signal (10% relative area) is assigned to the free aldehyde and the larger signal to the hydrate by analogy to the signal intensities of the two species in the proton NMR spectra [Chen et al. (1979) and spectra not shown]. Because the ^{19}F NMR signal intensity of macromolecules essentially disappears upon proton decoupling (resulting from a nuclear Overhauser effect, NOE, of −100%; Gerig, 1978; Sykes & Weiner, 1980), most spectra were obtained without proton decoupling. The apparent line width in the proton-coupled spectrum for the hydrate signal at −38.4 ppm and the free aldehyde signal at −37.6 ppm is 20–30 Hz, largely due to unresolved ortho and meta proton coupling to the fluorine nucleus.

Upon addition of Cht two new signals appear at −33.8 and −37.1 ppm in the ^1H-coupled ^{19}F NMR spectra (Figure 2). The signal at −37.1 ppm appears as a broad shoulder to the free aldehyde signal at −37.6 ppm. Association of the two new signals to the inhibitor–Cht complex is based upon (1) relative line widths, (2) signal intensities, and (3) signal disappearance upon decoupling. Both the −33.8- and −37.1-ppm signals are 20–30 Hz broader than the other two signals. The increased line widths for the signals of the enzyme-bound species are consistent with other ^{19}F NMR studies on Cht-bound inhibitors and are attributed to the slower tumbling of the macromolecular complex (Sykes & Weiner, 1980; Gerig, 1978). Also as shown in Figure 4, the percentage of the total ^{19}F signal area corresponding to the −33.8-ppm signal increases with increasing E_0/I_0 ratios. At very high ratios ($E_0/I_0 > 0.2$) the percent area of the −33.8-ppm signal reaches a saturation level of ~6% of the total ^{19}F signal area. The −37.1-ppm signal

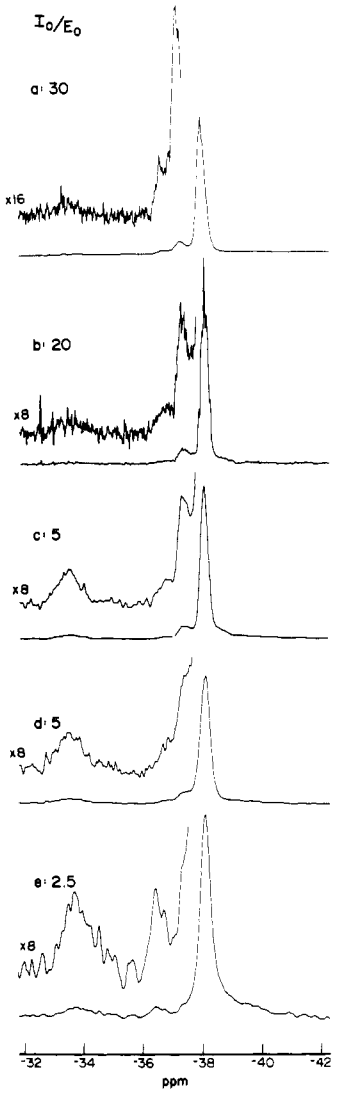


FIGURE 2: ^{19}F NMR spectrum of DL-*N*-AcFPheal in the presence of α-chymotrypsin at 25 °C, pD 6.1 ± 0.1, in 20% Me_2SO, with no ^1H decoupling and $[E]_0$ uncorrected for 85% active sites: (a) 60 mM aldehyde, 2.0 mM Cht; (b) 40 mM aldehyde, 2.0 mM Cht; (c) 20 mM aldehyde, 4.0 mM Cht; (d) 10 mM aldehyde, 2.0 mM Cht; (e) 5.0 mM aldehyde, 2.0 mM Cht. FID exponential multiplication of 1 Hz applied to (a) and (b), 4 Hz to (c) and (d), and 8 Hz to (e).

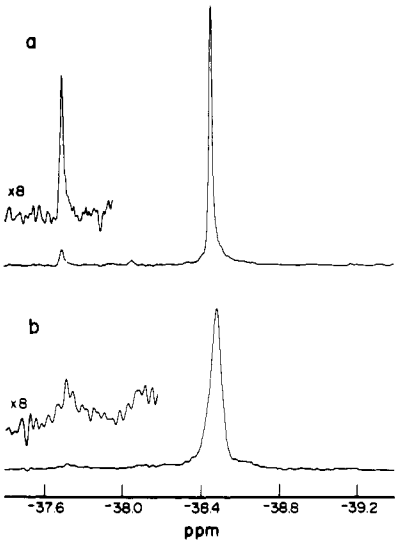


FIGURE 3: ^{19}F NMR spectrum of DL-*N*-AcFPheal in the presence of α-chymotrypsin at 25 °C and pD 6.2 with broad-band proton decoupling: (a) 60 mM aldehyde and no Cht; (b) 40 mM aldehyde and 1.7 mM Cht. Line broadening of 0.5 Hz to (a) and 0.2 Hz to (b).

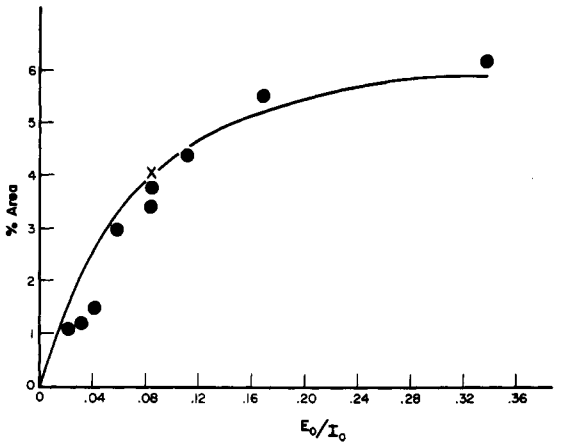


FIGURE 4: Plot of percentage area of hemiacetal signal at −33.6 ppm for DL-*N*-AcFPheal as a function of Cht/inhibitor ratio (E_0/I_0). Conditions: 25 °C, pD 6.1, $[E]_0$ = 1.7 (●) or 3.4 mM (×) ($[E]_0$ corrected for active sites), and pulsing rate of 2–3 s. The curve is drawn from the calculated percent hemiacetal by assuming K_H = 12 and K_i = 0.8 mM (Scheme II).

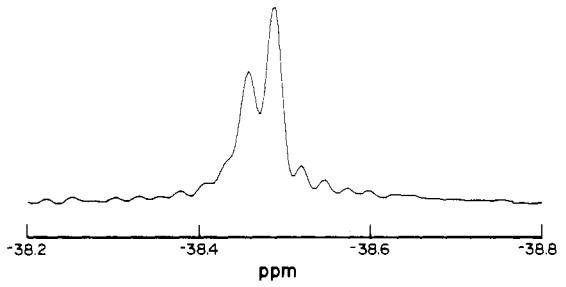


FIGURE 5: Decoupled ^{19}F NMR spectrum of 40 mM DL-AcFPheal and 1.7 mM Cht at 25 °C, pD 6.2. No line broadening was applied to the FID.

comprises 1.5–3% of the total signal area at higher E_0/I_0 ratios. Either gated broad-band or continuous broad-band decoupling of protons results in complete disappearance of both −33.8- and −37.1-ppm signals. In addition the free aldehyde signal at −37.6 ppm decreases dramatically in intensity relative to the hydrate signal. As shown in Figure 3 the free aldehyde signal at −37.6 ppm decreases from ~9% (Figure 3a) to 0.7% upon continuous broad-band ^1H irradiation (Figure 3b).

Continuous decoupling or decoupling for 0.1–1 s prior to and during signal acquisition (gated decoupling) results not only in a relative decrease in the aldehyde signal but also in a decrease in one of *two* observed hydrate signals. In the presence of 2 mM Cht the racemic mixture of *N*-AcFPheal is resolved into the D and L hydrate signals (Figure 5) at −38.426 and −38.484 ppm. The integration for the downfield signal at −38.426 ppm is 42%, for the upfield hydrate signal is 51%, and for the unresolved single aldehyde signal at −37.6 ppm is 7%. That the two hydrate signals separated by 0.06 ppm are attributable to the D and L enantiomers of the inhibitor is confirmed since only one hydrate signal is observed for the pure L-AcFPheal. Furthermore, addition of L-AcFPheal to a sample containing the racemate and Cht increases the signal intensity of the downfield signal, proving that the downfield hydrate signal is L-AcFPheal.

The L enantiomers of the aldehyde inhibitors are known to be better inhibitors than the D enantiomers (Chen et al., 1979; Table I), and it is the downfield L hydrate signal that is smaller (Figure 5). Gammon et al. (1972) have previously shown that

the D and L enantiomers of fluorinated *N*-acylphenylalanine can be resolved in the presence of Cht and that the more tightly bound D enantiomer is shifted downfield relative to the L inhibitor. This is exactly the opposite of our results for AcFPheal. In the *N*-acylfluorophenylalanines where the D enantiomer binds more tightly than the L enantiomer, it is believed that the α-carboxylate or *N*-acyl groups can interchange positions and bind in either enzyme locus near Ser-195 of the active site. (The aromatic ring in both enantiomers still binds in the hydrophobic specificity pocket.) In the aldehyde inhibitors, however, covalent binding is only possible for the L enantiomer so that the aldehyde carbon is in the α-carboxyl-Ser-195 locus. The weaker binding of the aldehyde D enantiomer reflects its inability to form a covalent complex.

The resolution of our hydrate signal would suggest that Cht stereospecifically binds the hydrate (at least the L enantiomer). However, our earlier ^{1}H NMR studies (Chen et al., 1979) and the above ^{1}H NMR results on the fluorinated aldehyde inhibitors indicate that the hydrate does *not* bind to Cht. The separation between the D and L hydrate signals is attributed instead to selective binding of the free L-aldehyde that is in chemical exchange with the *unbound* L hydrate. The signal at −37.1 ppm has been tentatively assigned to the bound aldehyde (assumed to be the L enantiomer). If the L hydrate and the bound L-aldehyde are in intermediate to fast exchange, then the L hydrate will be shifted somewhat downfield in the direction of the bound L-aldehyde. A 0.06-ppm shift is consistent with a chemical shift separation of 1.3 ppm between the hydrate and bound L-aldehyde where the maximum percentage concentration of the bound L-aldehyde is 4–10%. The decrease in intensity of the L hydrate relative to the D hydrate signal is consistent with this interpretation, since only enzyme-bound species or species in rapid chemical exchange with enzyme-bound species will experience a decrease in absolute and relative intensity upon ^{1}H irradiation due to the heteronuclear ^{19}F{^{1}H} negative NOE only for macromolecules. The L hydrate thus decreases in intensity relative to the D hydrate because of transfer of the NOE saturation of the bound L-aldehyde.

The furthest downfield signal at −33.8 ppm also disappears upon continuous proton irradiation. This signal, shifted about 4 ppm downfield from the other ^{19}F signals, is assigned to the bound L-hemiacetal and as a signal from a macromolecule is expected to be lost upon proton irradiation. In the coupled ^{19}F NMR spectra, the maximal concentration of the hemiacetal is 6% (Figure 4). Since the recycle time in these spectra is 2.5 s and since the T_1 relaxation time is much longer for the small molecule hydrate than that for any of the enzyme complex molecules, the hydrate signal will be selectively saturated at short recycle times. Thus the hydrate represents only 82% of the total ^{19}F signal at a 2.5-s recycle time, but 88% of the total signal at a 3.5-s recycle time. At very long recycle times, when the equilibrium magnetization is allowed to recover for all signals, the hemiacetal signal area will really be slightly less than 6%. Nevertheless, we will assume that the ^{19}F signal areas in these spectra give relatively reliable equilibrium concentrations for the various species.

We can calculate the percent hemiacetal signal assuming the following two equilibria:

Scheme II

$$\text{DL-A} \overset{K_H}{\rightleftharpoons} \text{DL-H}$$

$$\text{L-A} + \text{E} \overset{1/K_i}{\rightleftharpoons} \text{L-E•A}$$

H = hydrate, A = free aldehyde, and E·A represents both aldehyde Michaelis complex and hemiacetal–enzyme complex, only for the L enantiomer. This simplified scheme fails to distinguish between the two (observable) enzyme complexes and thus is technically deficient. However, considering the small amount of the noncovalent complex and errors associated with accurate concentration measurements from the signal areas, this simplification does not seem unreasonable. The curve drawn in Figure 4 is based upon scheme II, and the best fit of the data was obtained with $K_H = 12$ and $K_i = 0.8$ mM. Variation of K_H by ±2 and K_i by ±0.2 gave a noticeably poorer fit to the data. The K_H is essentially the same as the K_H (=11) measured in the absence of enzyme, but K_i is about 10 times larger than the value measured from inhibition kinetics (Table I; note the inhibition constant reported in Table I for L-AcFPheal should be divided by ~11 to correct for the fact that only the free aldehyde binds to the enzyme). This difference between the calculated and observed K_i's can probably be explained by enzyme dimerization (and oligomerization) at the NMR concentrations (Gammon et al., 1972), which is not a problem at the concentrations of Cht used in the kinetics. While it has been suggested (Gerig, 1977) that inhibitors bind equivalently to Cht dimers and monomers, our results suggest that the aldehydes have about a 10-fold poorer affinity for the Cht dimer than for the monomer. Propiolate in fact does bind less strongly to the Cht dimer than the monomer (Nichol et al., 1972). Gammon et al. (1972) have suggested that *N*-acyl-*p*-fluorophenylalanine binds *only* to the Cht monomer, which they indicate is the explanation for the 10-fold increase (0.3–3 mM) in K_i as $[E]_0$ varies from 0.05 to 1.9 mM. Our results suggest that inhibitors bind to both monomer and dimer (we could not otherwise explain the integrated intensity for the bound species in the ^{19}F NMR spectra) but bind with reduced affinity to the dimer.

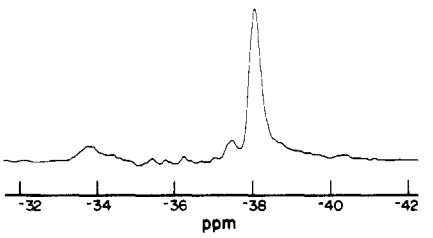


FIGURE 6: ^{19}F NMR spectrum of 9 mM DL-*N*-BzFPheal and 0.9 mM Cht, pD 6.2. An 8-Hz line broadening was applied to FID.

A K_i of less than 0.1 gives a very poor fit to the data since even at low inhibitor concentrations, the free aldehyde concentration is greater than K_i, the enzyme is nearly saturated, and the calculated percentage of E·A would be ~15%, rather than the ~6% observed. In fact, as shown in Figure 6, the ^{19}F area representing the hemiacetal complex of DL-BzFPheal is almost twice as large as the DL-AcFPheal hemiacetal signal. Since *N*-BzFPheal is a much better inhibitor than *N*-AcFPheal, with a K_i for the free aldehyde of ~6 μM, the benzoyl aldehyde will completely saturate the enzyme under these conditions.

The decrease in intensity of the free aldehyde signal at −37.6 ppm upon proton irradiation is also attributed to transfer of the NOE saturation of the bound aldehyde to the free aldehyde. The L-aldehyde must be in fast chemical exchange on the T_1 time scale (seconds) but in slow to intermediate chemical exchange on the chemical shift time scale with separate signals observed for the two states. The D-aldehyde likely also binds to Cht since nearly *all* of the free aldehyde signal can be saturated upon ^{1}H irradiation (Figure 3b). If only the L form bound, then only 50% of the free aldehyde signal should be saturable. We cannot say how much of the free aldehyde signal at −37.6 ppm arises from D or L enantiomers. However, there are no great differences between

signal areas in the spectra of L-AcFPheal and DL-AcFPheal in the presence of Cht. Recall, consistent with those results, Kennedy & Schultz (1979) have shown that the D enantiomer of BzPheal does bind to Cht although it is a much poorer inhibitor than the L enantiomer.

N-Benzoyl-DL-p-fluorophenylalaninal. The ^{19}F NMR spectra of BzFPheal bound to Cht are quite similar to the AcFPheal–Cht spectra (Figure 6). The bound BzFPheal hemiacetal at −34.0 ppm has approximately twice the intensity as the bound AcFPheal hemiacetal under similar conditions. No signal corresponding to the bound aldehyde form of AcFPheal is observed in the BzFPheal spectra. Thus part of the extra intensity of the hemiacetal form of BzFPheal could be attributable to an equilibrium in this case lying much more in favor of the hemiacetal than the bound aldehyde. (As discussed above, part of the extra intensity is also attributable to the smaller K_i.) In AcFPheal the equilibrium appears to be ~66:33 while in BzFPheal it must be >90:10. It is interesting that at pD 7.5, the −33.8-ppm signal for the *N*-acetyl aldehyde enzyme complex is maximally broadened (D. G. Gorenstein and D. O. Shah, unpublished results). Also at this pH the integration for the bound aldehyde signal at −37.7 ppm is 9%. At this higher pD where α-Cht is maximally active, the equilibrium is shifted even further to the bound hemiacetal.

These results could be a reflection of Jencks' "circe" effect, where binding energy is utilized to move the enzyme-bound species further along the reaction coordinate and stabilize transition-state structures. The benzoyl aldehyde inhibitors bind more tightly to Cht than the acetyl aldehyde inhibitors (Table I). The better benzoyl inhibitor binds entirely in the hemiacetal form, which represents a shift in the enzyme complex closer toward the transition-state structure.

Comparison with Other Fluorinated-Inhibitor–Enzyme Complexes. In previous fluorinated-inhibitor–Cht studies, chemical exchange between the free solution inhibitor and the enzyme-bound form was rapid on the NMR time scale and thus only a single averaged signal was observed (Sykes, 1969; Gammon et al., 1972; Tsavalos et al., 1978). Neither the chemical shift nor line widths of the separate ^{19}F NMR signals are dependent upon the E_0/I_0 ratios in our study. This is consistent with slow chemical exchange of our inhibitor between enzyme-bound forms. However, as discussed earlier, the hydrate and bound aldehyde are likely in more rapid chemical exchange. (The effects of this exchange will be quite small.) The aldehyde inhibitors are more tightly bound than substratelike or productlike inhibitors as reflects a "transition-state analogue". The dissociation rate constant will be smaller, and with the ~4-ppm separation between signals, the observation of slow exchange is quite reasonable.

The downfield shift of the ^{19}F signal of an enzyme-bound species is consistent with other studies. The ^{19}F signals of various *p*-fluorophenylalanine inhibitors are shifted 1–2 ppm downfield upon binding to Cht (Tsavalos et al., 1978; Knight et al., 1978). Current ^{19}F NMR chemical shift theory attributes these downfield shifts to van der Waals interactions with protein protons and thus to binding of the *p*-fluorophenyl ring into the aromatic binding pocket. Hull & Sykes (1976) and Gerig (1978) have demonstrated that as an ^{19}F signal is shifted further downfield, the more tightly immobilized and buried the aromatic ring is in the protein complex (the protein-induced shift is roughly proportional to $1/r^6$, where r is the internuclear distance between interacting nuclei). The ~4-ppm downfield shift we observe for BzPheal and AcFPheal is one of the largest protein-induced shifts observed for a reversibly bound inhibitor to Cht. The only comparable enzyme-induced shift is found for the *covalently* bound complexes *p*-fluorobenzoyl–Cht (Amshey, 1973), *p*-fluorocinnamoyl–Cht, (Gerig & Halley, 1981), and *p*-fluorobenzenesulfonyl–Cht (Maddox et al., 1975).

In contrast, the bound *N*-AcFPheal signal is shifted <1 ppm downfield from the free aldehyde. This suggests that the Michaelis aldehyde–enzyme complex has the aromatic ring only loosely buried in the aromatic binding pocket, while in the hemiacetal complex this ring must be more highly immobilized. It is not known whether this reflects a true conformational change of the enzyme and an induced fit of the complex. Gerig & Halley (1981) have also noted that the binding of the aromatic ring in noncovalent complexes differs considerably from the covalent acyl enzymes. Krieger et al. (1974) have, in fact, observed that binding of benzamidine to trypsin induces a "squeeze" in the specificity binding pocket. Formation of the hemiacetal, transition-state analogue complex likely induces a similar tightening of the aromatic binding pocket.

References

Amshey, J. W., Jr. (1973) Ph.D. Dissertation, Northwestern University.

Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T., & Umezawa, H. (1969) *J. Antibiot. 22*, 558.

Bender, M. L., & Kemp, K. C. (1957) *J. Am. Chem. Soc. 79*, 116.

Breaux, E. J., & Bender, M. L. (1975) *FEBS Lett. 56*, 81.

Chen, R., Gorenstein, D. G., Kennedy, W. P., Lowe, G., Nurse, D., & Schultz, R. M. (1979) *Biochemistry 18*, 921.

Clark, P. I., Lowe, G., & Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 451.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Dixon, M., & Webb, E. C. (1964) *Enzymes*, 2nd ed., p 327, Academic Press, New York.

Gammon, K. L., Smallcombe, S. H., & Richards, J. H. (1972) *J. Am. Chem. Soc. 94*, 4573.

Gerig, J. T. (1977) *J. Am. Chem. Soc. 99*, 1721.

Gerig, J. T. (1978) in *Biochemical Magnetic Resonance* (Berliner, L. J., & Reuben, J., Eds.) Chapter 5, Plenum Press, New York.

Gerig, J. R., & Halley, B. A. (1981) *Arch. Biochem. Biophys. 43*, 427.

Gorenstein, D. G., Kar, D., & Momii, R. K. (1976) *Biochem. Biophys. Res. Commun. 73*, 105.

Hull, W. E., & Sykes, B. D. (1976) *Biochemistry 15*, 1535.

Ito, A., Tokawa, K., & Shimizu, B. (1972) *Biochem. Biophys. Res. Commun. 49*, 343.

Ito, A., Tamahashi, R., & Baba, Y. (1975) *Chem. Pharm. Bull. 23*, 3081.

Kawamura, K., Kondo, S., Maeda, K., & Umezawa, H. (1969) *Chem. Pharm. Bull. 17*, 1902.

Kennedy, W. P., & Schultz, R. M. (1979) *Biochemistry 18*, 349.

Knight, H. J., Williams, E. H., & Spotswood, T. M. (1978) *Aust. J. Chem. 31*, 2187.

Kondo, S., Kawamura, K., Iwanaga, J., Hanada, M., Aoyagi, T., Maeda, K., Takeuchi, T., & Umezawa, H. (1969) *Chem. Pharm. Bull. 17*, 1896.

Krieger, M., Kay, L., & Stroud, R. M. (1974) *J. Mol. Biol. 83*, 209.

Lewis, C. A., Jr., & Wolfenden, R. (1977) *Biochemistry 16*, 4890.

Lienhard, G. E. (1972) *Annu. Rep. Med. Chem. 7*, 249.

Lienhard, G. E. (1973) *Science (Washington, D.C.) 180*, 149.

Lowe, G., & Nurse, D. (1977) *J. Chem. Soc., Chem. Commun.*, 815.
Maddox, M. L., Roe, D. C., & Gerig, J. T. (1975) *J. Chem. Soc., Chem. Commun.*, 902.
Nichol, L. W., Jackson, W. J. H., & Winzor, D. J. (1972) *Biochemistry 11*, 585.
Pauling, L. (1946) *Chem. Eng. News 24*, 1375.
Schonbaum, G. R., Zerner, B., & Bender, M. L. (1961) *J. Biol. Chem. 236*, 2930.
Schultz, R. M., & Cheerva, A. C. (1975) *FEBS Lett. 50*, 47.
Sykes, B. D. (1969) *J. Am. Chem. Soc. 91*, 949.
Sykes, B. D., & Weiner, J. H. (1980) in *Magnetic Resonance in Biology* (Cohen, J. S., Ed.) Vol. 1, pp 171-196, Wiley, New York.
Thompson, R. C. (1973) *Biochemistry 12*, 47.
Thompson, R. C. (1974) *Biochemistry 13*, 5495.
Thompson, R. C. (1977) *Methods Enzymol. 46*, 220.
Tsavalos, M., Nicholson, B. C., & Spotswood, T. M. (1978) *Aust. J. Chem. 31*, 2179.
Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10.
Wyeth, P., Sharma, R. P., & Akhtar, M. (1980) *Eur. J. Biochem. 105*, 581.

Kinetics for Exchange of Imino Protons in Deoxyribonucleic Acid, Ribonucleic Acid, and Hybrid Oligonucleotide Helices†

Arthur Pardi*,‡ and Ignacio Tinoco, Jr.

ABSTRACT: The lifetimes for opening of individual base pairs in a DNA ($dCA_5G + dCT_5G$), an RNA ($rCA_5G + rCU_5G$), and a hybrid DNA–RNA ($rCA_5G + dCT_5G$) helix have been measured by proton nuclear magnetic resonance. The lifetimes were obtained by saturation recovery experiments performed on the hydrogen-bonding imino protons of the Watson–Crick base pairs. In these oligonucleotide helices the observed relaxation rates were dominated by exchange with water, with the magnetic spin–lattice relaxation time of the imino protons possibly being important only at the lowest temperatures in the DNA helix. It was shown that three interior base pairs in the DNA heptamer $dCA_5G + dCT_5G$ were in the open-limited region, which means that these imino protons exchange every time the base pair opens. The lifetimes of the terminal G·C base pairs in the DNA helix are much shorter than the interior A·T base pairs. The pH dependence of the terminal base pairs indicated that the ends of the helix open and close many times before exchange of the imino protons with water takes place. The temperature dependence of the lifetimes of the interior A·T imino protons in the DNA helix showed that these protons exchange only when the double helix has dissociated into single strands. Thus, these lifetimes measure the rate for dissociation of the double helix. The activation energy for this process was found to be 47 kcal/mol. Comparison of the lifetimes of the interior protons in the DNA, RNA, and hybrid helices showed that the rates of dissociation of the RNA and hybrid helices are very similar at 5 °C, whereas the rate for the DNA helix was approximately 1 order of magnitude smaller than that for the other two helices. The reasons for the differences in the kinetics of the three helices are discussed, as are the general dynamics of oligonucleotide helices in solution.

In double-helical nucleic acids the base-paired imino protons exchange slowly enough with water to be observed in nuclear magnetic resonance (NMR)[1] experiments. These protons were first studied in solution studies of tRNA and also in double-helical oligonucleotides [see Kearns (1977) and Robillard & Reid (1979) for reviews]. The chemical shift of an imino proton in a base pair depends upon its intrinsic shift and an interaction term that arises mainly from the ring currents of the neighboring base pairs in the sequence. As the interaction term is dependent upon conformation, most of the information obtained from the NMR of the imino protons of tRNAs and oligonucleotides has been used to study the conformation of these molecules in solution (Robillard & Reid, 1979; Kearns, 1977).

Crothers et al. (1974) used NMR to study the dynamics of *Escherichia coli* $tRNA^{fMet}$ by watching the broadening of the imino protons with temperature. Kinetic measurements obtained by this method are very limited in the range of lifetimes that can be observed. Johnston & Redfield (1977) applied saturation recovery experiments to the imino protons to determine the exchange rates with water of the individual imino protons in tRNA. Other workers used this technique to study the helix–coil dynamics of tRNA (Hurd & Reid, 1980). Early et al. (1981a,b) have recently used a long-pulse inversion recovery experiment to study the relaxation behavior of several DNA restriction fragments, and in a following paper we have used saturation recovery techniques to study the dynamics of several related DNA oligonucleotides in solution (A. Pardi, K. M. Morden, D. J. Patel, and I. Tinoco, Jr., unpublished results). Thus proton NMR is now beginning to show its usefulness in obtaining information on the dynamics, as well as the conformations, of nucleic acids in solution.

The kinetics of helix–coil transitions in oligonucleotides has mainly been studied by using relaxation techniques, such as temperature-jump experiments (Pörschke & Eigen, 1971;

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

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