Structure of Methanofuran, the Carbon Dioxide Reduction Factor of *Methanobacterium thermoautotrophicum*

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Abstract: The structure of the carbon dioxide reduction factor from *Methanobacterium thermoautotrophicum* has been assigned by analysis of the intact molecule and its hydrolytic fragments as 4-[*N*-(4,5,7-tricarboxyheptanoyl-γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan. High-resolution NMR spectroscopy and FAB, FD, and EI mass spectrometry were used, with FABMS being decisive. The trivial name methanofuran is proposed for the carbon dioxide reduction factor.

The carbon dioxide reduction factor is a novel coenzyme required for methane formation from hydrogen gas and carbon dioxide[2,3] in extracts of the methanogenic bacterium *Methanobacterium thermoautotrophicum*. The methanogenic bacteria, members of the distinct group archaebacteria,[4,5] have been shown to contain several coenzymes that were not previously known.[4,6] Since the carbon dioxide reduction factor could not be replaced by known coenzymes in its ability to stimulate methane production,[3] its structure was of interest. Its purification and initial characterization have been described.[3] We now assign it the structure shown in Chart I and hereafter give it the descriptive name methanofuran.

The molecular weight of methanofuran was found to be 748 (M + H = 749) by positive ion fast atom bombardment mass spectrometry (+ ion FABMS).[7] Data from high-resolution FABMS and elemental analyses indicated the molecular formula $C_{34}H_{44}N_4O_{15}$.

Fragments of methanofuran were obtained by acidic hydrolysis in 1 N hydrochloric acid and by alkaline hydrolysis in 1 N potassium hydroxide, both at 100 °C. The acidic hydrolysis was more productive, giving fragments AEP, F1, F3, F4, F5, and TCA (see Chart I for the location of the fragments in methanofuran). Alkaline hydrolysis yielded ALK 1.

The easiest fragment to identify was AEP. The ^{1}H NMR spectra of methanofuran (Figure 1) had already shown four two-proton groups, a four-proton AB quartet at 6.98 and 7.21 ppm (J = 9 Hz, decoupled by homonuclear decoupling) indicating a para-disubstituted benzene, and triplets at 2.75 and 3.42 ppm (J = 7 Hz, again decoupled by homonuclear decoupling) indicating adjacent methylene groups. These protons were also found in AEP (see Experimental Section). Comparison of the electron ionization (EI) mass spectrum and ^{1}H NMR spectrum of AEP with published spectra[8,9] established the identity of AEP as *p*-(β-aminoethyl)phenol.

The ^{1}H NMR spectrum of fragment F4 (in D_2O) contained the signals of AEP plus an additional one-proton triplet at 3.73 ppm and protons for two additional methylene groups at 2.36 and 2.05 ppm. Similar signals were observed in the ^{1}H NMR spectrum of fragment F5 (Table I), as well as another set of protons similar to the non-AEP protons of F4, consisting of a one-proton multiplet and two methylenes, with the highest field protons (near 2 ppm) consisting of two one-proton signals rather than one two-proton signal. FAB mass spectra of F4 and F5 showed molecular weights of 266 and 395, respectively, increments of 129 and 258 amu over the molecular weight of AEP. These data were consistent with structures for F4 and F5 containing AEP plus one or two glutamic acid (Glu) residues, respectively. Proton homonuclear decoupling on F5 at pH 7 (Table I) confirmed a pattern corresponding to $>CHCH_2CH_2-$ for each proposed Glu residue. The β-methylene protons of one Glu residue in F5 were magnetically nonequivalent. We have observed a similar magnetic nonequivalence of the two β-methylene protons of Glu in the ^{1}H NMR spectrum of folic acid; these are found at 2.17 and 2.04 ppm.[10]

To confirm the presence of Glu and establish its chirality, *N*-trifluoroacetyl methyl esters of amino acids obtained from a

Chart I

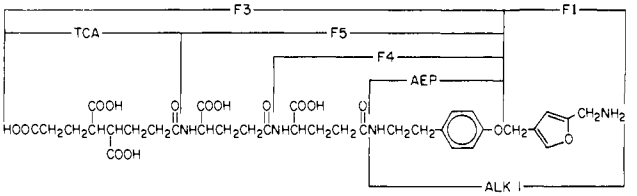

Table I. ^{1}H NMR Signals for F5

assignment[a]	δ[b] pH 7.2	δ[b] pH 1.5	multiplicity,[c] intensity	change on irradiation (pH 7.2)
AEP				
H-2	6.86	6.85	d, 2 H	
H-3	7.16	7.16	d, 2 H	
H-1′	2.75	2.75	t, 2 H	
H-2′	3.41	3.41	m, 2 H	
Glu_1				
α_1	4.09	4.24	m, 1 H	
β_1	2.02	2.07	m, 1 H	
β_1	1.83	1.91	m, 1 H	4.09 → s, 2.21 → d
γ_1	2.21	2.26	t, 2 H	
Glu_2				
α_2	3.78	4.05	t, 1 H	
β_2	2.14	2.22	m, 2 H	3.78 → s, 2.47 → s
γ_2	2.47	2.53	t, 2 H	

[a] α, β, and γ indicate the type of glutamic acid protons; subscript distinguishes the two glutamyl moieties, with Glu_1 being attached to AEP. [b] Solvent D_2O with sodium phosphate buffer; chemical shifts relative to TSP (0.00 ppm). [c] s = singlet, d = doublet, t = triplet, m = multiplet.

(1) (a) Department of Microbiology. (b) Department of Chemistry.
(2) Romesser, J. A.; Wolfe, R. S. *Zentralbl. Bakteriol., Mikrobiol. Hyg., Abt. 1, Orig. C* **1982**, *3*, 271-276.
(3) Leigh, J. A.; Wolfe, R. S. *J. Biol. Chem.* **1983**, *258*, 7536-7540.
(4) Balch, W. E.; Fox, G. E.; Magrum, L. J.; Woese, C. R.; Wolfe, R. S. *Microbiol. Rev.* **1979**, *43*, 260-296.
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(6) Wolfe, R. S. *Antonie van Leeuwenhoek* **1979**, *45*, 353-364.
(7) Rinehart, K. L., Jr. *Science (Washington D.C.)* **1982**, *218*, 254-260.
(8) "EPA/NIH Mass Spectral Data Base;" Heller, S. R., Milne, G. W. A., Eds.; U.S. Government Printing Office: Washington, D.C.; 1978; Vol. 1, p 325.
(9) "Nuclear Magnetic Resonance Spectra" (Sadtler Standard Spectra); Sadtler Research Laboratories: Philadelphia, 1968; Spectrum No. 3239.
(10) J. A. Leigh, unpublished observations.

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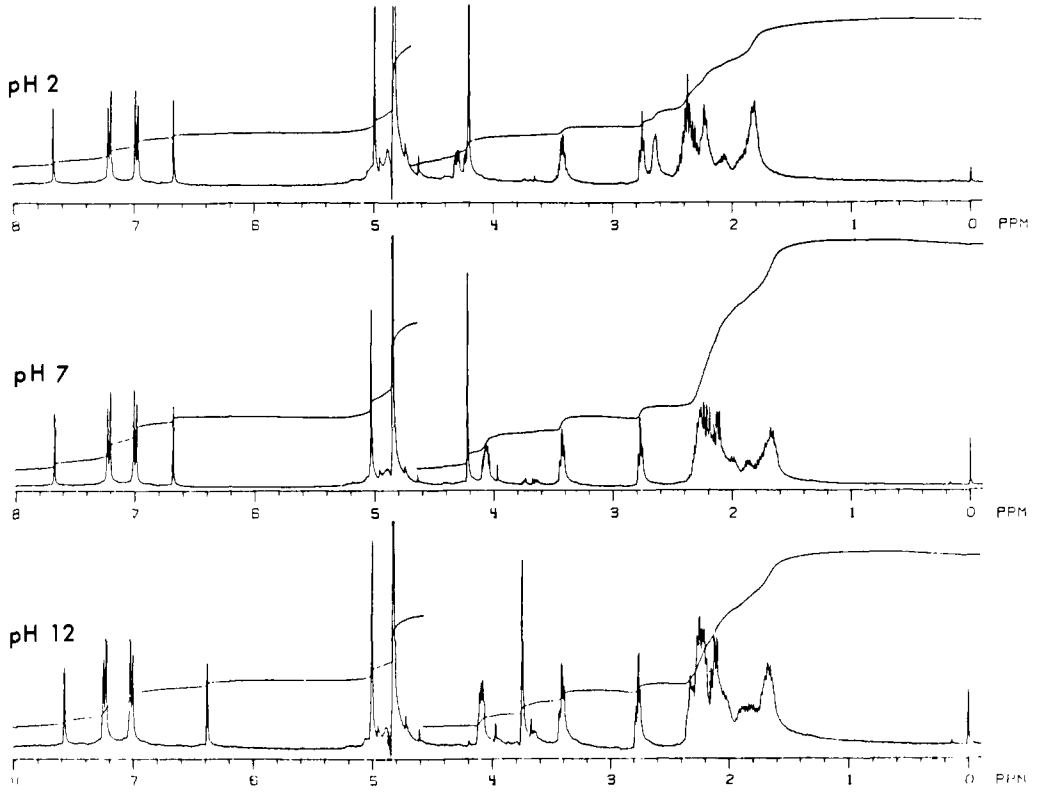

Figure 1. ^{1}H NMR spectra of methanofuran at different pH values in solvent D_2O with sodium phosphate buffer; chemical shifts relative to TSP (0.00 ppm).

total hydrolysis of methanofuran were separated by gas chromatography (GC) on an optically active column: L-Glu, but not D-Glu, was present. Shift of the 2′-protons (adjacent to nitrogen) in going from AEP to F4 (3.24 → 3.43) locates Glu_1 as being attached to the β-amino group of AEP. Similarly, shift of the α_1-proton of Glu in going from F4 to F5 (3.73 → 4.09) locates Glu_2 as being attached to the amino group of Glu_1, i.e., as Glu-Glu-AEP in F5. Evidence that both Glu residues were linked by their γ-carboxyls came from the observation that acidification caused the methine protons (α-protons) in the ^{1}H NMR spectrum of F5 (Table I) to shift more than the γ-methylene protons. Results of homonuclear decoupling confirmed the relationships among the protons in the acidified spectrum in the same way that is shown in Table I for the protons in the spectrum at neutral pH. In addition, both F4 and F5 (0.1 mg) formed deep orange cobalt(II) Schiff base complexes with pyridine-2-carboxaldehyde, characteristic of α-amino acids.[11]

FABMS showed the molecular weight of fragment F3 to be 639, and HRFABMS and elemental analyses indicated the molecular formula $C_{28}H_{37}N_3O_{14}$, i.e., F5 with an attached $C_{10}H_{13}O_7$ unit. The ^{1}H NMR spectrum of F3 in dimethyl-d_6 sulfoxide (Me_2SO-d_6) showed all the signals of F5 as well as additional complex resonances in the high-field region (see Experimental Section) and three additional signals at 7.85 (t), 8.12 (d), and 8.20 ppm (d) which disappeared with the addition of deuterium oxide. Irradiating at 3.15 ppm collapsed the triplet at 7.85 ppm to a singlet; these, then, were the signals for $-NHCH_2-$ of the *p*-(β-aminoethyl)phenol group. Irradiating at 4.12 ppm collapsed the two doublets at 8.12 and 8.20 ppm to singlets and simplified the complex signals at 1.95 and 1.65 ppm. Therefore, the signals at 8.12 and 8.20 ppm appeared to be the amide hydrogens of the glutamyl groups, and the signal at 4.12 ppm the methine protons of the two glutamyl groups. A shift in the UV spectrum of F3 at basic pH argued the presence of a free phenolic group.

The remainder of F3, which yielded the additional signals in the high-field region of the ^{1}H NMR spectra, was accounted for by the tetracarboxylic acid (TCA) fragment which appeared to be the difference between F5 and F3. Field desorption (FD)MS of TCA indicated the expected molecular weight of 262, and it followed from the molecular formula of F3 and its known constituents that the molecular formula for TCA was $C_{10}H_{14}O_8$. The ^{1}H NMR spectrum of TCA showed protons in the high-field region, indicating a high degree of symmetry and accounting for the differences between the ^{1}H NMR spectra of F5 and F3. Three complex signals were present. Irradiating at 1.91 ppm (4 H) simplified the signals at 2.74 (2 H) and 2.49 ppm (4 H) to a singlet and an AB quartet, respectively. These results, together with the integral ratios (1:2:2), indicated a symmetrical molecule containing two $>CHCH_2CH_2-$ units, with some deshielding of the protons on the outer carbons. To incorporate the remaining $C_4H_4O_8$ residue (four carboxyl groups), the structure 4,5-dicarboxyoctanedioic acid is assigned. No circular dichroism was detected for TCA (0.5 mg, carried out to 200 nm), arguing meso stereochemistry, i.e., (4*R*,5*S*)-4,5-dicarboxyoctanedioic acid.

Evidence that TCA is linked to Glu by a terminal carboxyl was found by comparing the ^{1}H NMR spectra of methanofuran at pH 2 and 7 (Figure 1, top and middle, respectively). Integration showed that the peak which was shifted upfield to 2.65 ppm by acidification represents two protons. Of all the protons in this region (2–3 ppm) that could be affected by protonation of an acidic group (methines or methylenes of TCA or methylenes of Glu residues), these are the farthest downfield, which would be the methines in the 4 and 5 positions of TCA. Since two protons were shifted (rather than one), both carboxyls in the 4 and 5 positions must be free. Because TCA is meso, the carboxyl attached to Glu_2 can be C-1 or C-8, closer either to the *R* or to the *S* asymmetric center of TCA; it is not known at present which is the case.

The remaining fragment from acidic hydrolysis of methanofuran was F1, whose HREI mass spectrum (10 and 70 eV) indicated that its molecular formula was $C_6H_9NO_2$ (Δ -0.2 mmu), agreeing with that predicted by the difference between methanofuran and F3. The ^{1}H NMR spectra of F1 in deuterium oxide and deuteriochloroform showed two olefinic or aromatic one-proton singlets and two methylene singlets at rather low field. The ^{1}H NMR spectrum in Me_2SO-d_6 contained another broad peak at 8.24 ppm

(11) Feigl, F. "Spot Tests in Organic Analysis"; Elsevier: New York, 1966; p 370.

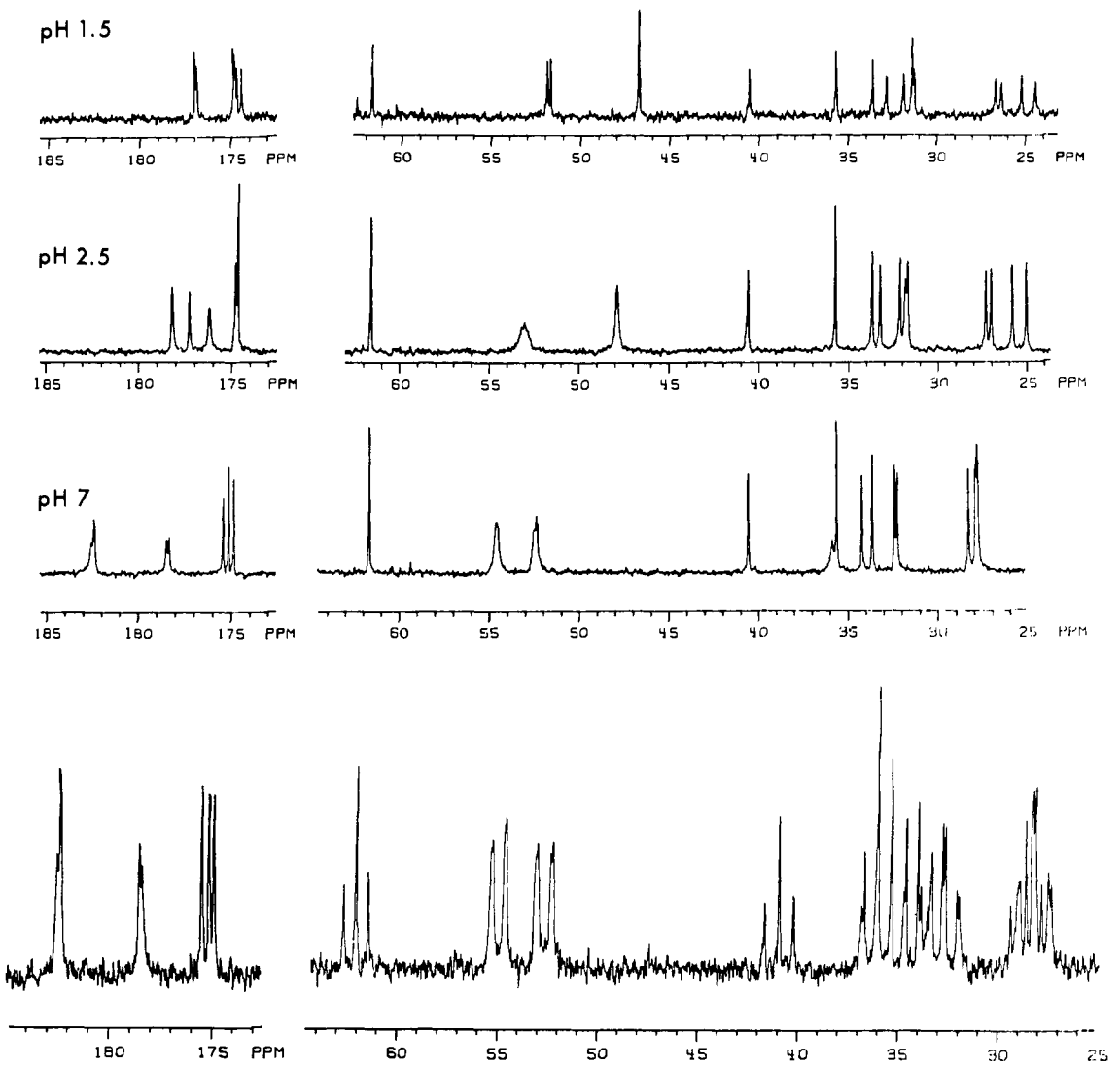

Figure 2. ^{13}C NMR spectra of methanofuran: solvent D_2O; chemical shifts relative to 1,4-dioxane (66.5 ppm); pH adjusted with KOH or HCl for the top three, broad band decoupled spectra; the bottom spectrum is off-resonance decoupled. Resonances between 64 and 173 ppm are reported in the Experimental Section.

and an additional one-proton triplet at 4.99 ppm; also, one of the methylenes (4.23 ppm) was a doublet. When deuterium oxide was added, the peaks at 8.24 and 4.99 ppm disappeared, the doublet at 4.23 ppm collapsed to a singlet, and the methylene peak at 3.98 ppm increased in intensity. Irradiating at 4.99 ppm collapsed the doublet at 4.23 ppm to a singlet and irradiating at 4.23 ppm collapsed the triplet at 4.99 ppm to a singlet. Therefore, the methylene at 4.23 ppm is coupled to an exchangeable single proton and the methylene at 3.98 ppm may be coupled to two or three exchangeable protons represented by the broad peak at 8.24 ppm. In the ^1H NMR spectrum in deuterium oxide, the attachment of the higher field methylene to an amino group was evident from its shift from 4.21 to 3.76 ppm at basic pH. Similar shifts with pH were seen in the ^1H NMR spectrum of methanofuran (Figure 1). Treatment of F1 with acetic anhydride resulted in acetylation of the groups associated with both methylenes with accompanying downfield shifts (in $CDCl_3$), from 4.52 to 4.92 ppm and from 3.80 to 4.42 ppm for acetylated F1 compared to F1. The lower field methylene of F1, then, is attached to a hydroxyl, and since the methylene peak at 4.42 ppm in the spectrum of acetylated F1 was a doublet, collapsible by irradiation at 5.75 ppm, the other must be a $-CH_2NH_2$ group.

In addition to the hydroxymethyl and aminomethyl groups, the molecular formula of F1 and the olefinic protons would accommodate a 2,4-disubstituted furan. Comparison of the ^1H NMR spectrum of F1 with published spectra of furan, furfurylamine, and furfuryl alcohol in deuteriochloroform[12] led to the conclusion that F1 is 4-(hydroxymethyl)furfurylamine rather than 4-(aminomethyl)furfuryl alcohol. The attachment of a hydroxymethyl group causes only very slight changes in the positions of the aromatic protons (H-3, H-4, and H-5 at 6.33, 6.33, and 7.44 ppm in furfuryl alcohol vs. H-3 and H-5 at 6.37 and 7.42 ppm in furan), while attachment of an aminomethyl group causes larger upfield shifts, especially at adjacent hydrogens (H-3, H-4, and H-5 at 6.13, 6.30, and 7.33 ppm in furfurylamine). Thus, since H-5 in F1 appears at exactly the same position as in furfurylamine, the structure should be that of a 4-(hydroxymethyl)furfurylamine. In agreement with this assignment, H-3 is shifted much more (6.60 → 6.31) at higher pH than is H-5 (7.58 → 7.47).

The linkage of F1 to F3 in methanofuran can be assigned as an ether linkage, since it resists cleavage by alkali. The alkaline hydrolysis fragment ALK 1 was analyzed by EIMS and ^1H NMR spectroscopy. Its molecular weight, 246, was consistent with a compound representing the condensation of F1 and AEP, and HREIMS confirmed the molecular formula as $C_{14}H_{18}N_2O_2$ (measured m/z 246.1351, Δ 1.7 mmu). The expected signals were present in the ^1H NMR spectra of ALK 1. In deuterium oxide the methylene protons of the oxymethyl group of F1 were shifted downfield by attachment to the substituted phenyl group, as also observed in methanofuran (Figure 1). The two primary amines

(12) Bhacca, N. S.; Johnson, L. F.; Shoolery, J. N. "NMR Spectra Catalog" (Varian Instrument Division); Varian Associates: Palo Alto, 1962; Spectra No. 50, 102, and 104.

appeared in Me_2SO-d_6 as broad low-field peaks which disappeared on addition of deuterium oxide.

The proposed structure of methanofuran was further confirmed by ^{13}C NMR spectroscopy (Figure 2 and Experimental Section). In the aromatic region, the expected four tertiary and four quaternary carbon signals were observed, belonging to the para-substituted phenyl and the 2,4-disubstituted furan. Seven carboxyl absorptions were observed at pH 7; presumably the eighth carboxyl was part of the broad signals near 179 and 183 ppm. The methylene signals at 61.7 and 40.6 ppm (Figure 2) were assigned to the oxymethyl and aminomethyl groups of the 2,4-disubstituted furan, respectively.[13] Four methine carbons were observed between 52 and 55 ppm, corresponding to the two methines of the TCA unit and the two methines of the Glu residues. Ten methylene carbons were found between 27 and 36 ppm, four belonging to the TCA unit, four to the two Glu's, and two to AEP.

Results of ^{13}C NMR spectroscopy of methanofuran at different pH's (Figure 2) supported the proposed linkages of the Glu and TCA groups in the molecule. The upfield shifts of the methine carbons on acidification (pH 7 → 1.5) were 2.8, 3.0, 5.5, and 5.7 ppm. The differences in chemical shifts reported for pentanoic acid and its ammonium salt are 3.9 ppm for the α-methylene carbon and 0.6 ppm for the γ-methylene carbon.[14] The observed shifts in methanofuran thus indicate that all four methine carbons are α to free carboxyls rather than γ.

In conclusion, the present study has assigned the structure of methanofuran, with accompanying stereochemistry. The molecule is unique among cofactors of reducing pathways, differing greatly, for example, from folic acid, though both contain Glu units. Results presented elsewhere[15] indicate that in methanogenesis, from carbon dioxide, a formyl group binds to the furfurylamine site on methanofuran. Studies on the chemical synthesis are in progress.

Experimental Section

General. Low-resolution electron ionization (EI) mass spectra were determined on a Finnigan MAT mass spectrometer, Model CH-5 DF, by using the direct probe technique. Fast atom bombardment (FAB) mass spectra, field desorption (FD) mass spectra, and high-resolution (HR) mass spectra were obtained on a Finnigan MAT mass spectrometer, Model 731, or a VG Analytical mass spectrometer, Model 7070. Samples for FABMS were dissolved in water and suspended in a glycerol matrix. HREI spectra were obtained by peak matching or computer scan, HRFAB and HRFD spectra by peak matching, both at a resolution of 8000–10 000. Nuclear magnetic resonance (NMR) spectra were obtained on a Nicolet 360-MHz Fourier transform spectrometer equipped with vertical probes for 5-mm tubes for both ^1H and ^{13}C spectroscopy. For ^1H NMR spectra, the observed frequency was 360.061 MHz, the sweep width 2000 Hz, the pulse width 3 μs, and the postacquisition delay 250 μs. For ^{13}C NMR spectra, the observed frequency was 90.546 MHz, the sweep width 10 000 Hz, the pulse width 2 μs, the postacquisition delay 1 s, and the proton 90° pulse 90 μs. The decoupler frequency was 360.061 MHz for fully proton-decoupled spectra and 360.063 MHz for off-resonance decoupling. Fully proton-decoupled spectra were obtained with Levitt–Freeman cycle decoupling. The proton 180° pulse was 180 μs. The chemical shift standards were 3-(trimethylsilyl)propionic acid (TSP) or tetramethylsilane (Me_4Si) (0.00 ppm, ^1H NMR) and 1,4-dioxane (66.5 ppm, ^{13}C NMR).

Ultraviolet (UV) spectra were obtained on a Cary Model 14 scanning spectrophotometer. High-performance liquid chromatography (HPLC) was carried out by using Waters pumps, Model 6000 A, a Waters differential refractometer detector, Model R401, a Kratos multiwavelength UV detector, Model SF740 (Schoeffel Instrument Div., Westwood, NJ), and a Waters solvent programmer, Model 660. Gas chromatography (GC) was performed by using a Varian chromatograph, Model 3700, equipped with a flame ionization detector. Circular dichroism (CD) measurements were carried out at The Upjohn Co. in methanol at room temperature on a Jasco instrument, Model 500-C.

Methanofuran was isolated by the procedure described earlier.[3] It had the following spectroscopic properties: FABMS (+ ion) m/z (relative intensity) 801 (6), 799 (16, M + CH_2 + H + HCl),[16] 787 (8) and 785 (18, M + H + HCl), 777 (16, M + 2 × CH_2 + H),[16] 765 (12), 764 (36), 763 (80, M + CH_2 + H),[16] 751 (15), 750 (45), 749 (100, M + H), 732 (11); ^1H NMR, see Figure 1; ^{13}C NMR, see Figure 2, as well as signals at δ 155.9 (s), 147.2 (s), 143.0 (d), 132.4 (s), 130.1 (d), 121.9 (s), 115.2 (d), 111.3 (d); calcd for $C_{34}H_{44}N_4O_{15}$, M_r 749.2881 (M + H); found, M_r 749.2881 (M + H, HRFABMS).

Acidic Hydrolysis of Methanofuran. Methanofuran (8 mg) was hydrolyzed in 1 N hydrochloric acid (0.5 mL) at 100 °C for 15 min in an evacuated, sealed glass ampule. The hydrolysate was dried at room temperature in a vacuum oven containing sodium hydroxide pellets, redissolved in water, and allowed to sit overnight at room temperature. Fragments were purified by HPLC on a Waters μBondapak C_{18} column (7.8 mm × 30 cm, 10 μm) at a flow rate of 4 mL/min by using an isocratic elution with water (60 mL) followed by a 30-min (120 mL) linear gradient from 0 to 70% methanol, both employing 1% formic acid adjusted to pH 3 with ammonium hydroxide. The acidic hydrolysis fragments F1, AEP, and TCA were detected by refractive index (attenuation 4×) and eluted before the start of the methanol gradient at 16, 32, and 48 mL, respectively, after injection. The acidic hydrolysis fragments F4, F5, and F3 were detected by UV absorbance at 215 nm (absorbance range 0.5) and eluted at 32, 44, and 60 mL, respectively, after the start of the gradient. Purified fragments were dried and desalted by rotary evaporation, then redissolved in water and lyophilized. For mass spectrometry, methanofuran and its fragments were further desalted by HPLC as follows: A Waters μBondapak C_{18} column (7.8 mm × 30 cm, 10 μm) was equilibrated by flushing with 1 mM hydrochloric acid followed by 1 mM hydrochloric acid in 70% methanol and again with 1 mM hydrochloric acid. Samples were injected, eluted with a gradient (0–70% methanol), and detected by UV absorbance at 215 nm. The fragments had the following spectroscopic properties.

AEP: EIMS (70 eV) m/z (relative intensity) 137 (10, M), 108 (55), 107 (30), 77 (13), 51 (7), 44 (10), 39 (7), 38 (8), 36 (23), 30 (100); ^1H NMR (CF_3COOD; TSP, 0.00) δ 6.90 (d), 6.70 (d), 3.23 (t), 2.78 (t) (all 2 H); ^1H NMR (D_2O) δ 7.22 (d), 6.90 (d), 3.24 (t), 2.92 (t) (all 2 H); UV (H_2O) λ_{max} 220, 273, 280 nm (sh).

F4: FABMS (+ ion) m/z (relative intensity) 359 (8, M + H + glycerol), 289 (9, M + Na), 268 (20), 267 (100, M + H); ^1H NMR (D_2O) δ 7.17 (2 H, d), 6.86 (2 H, d), 3.73 (1 H, t), 3.43 (2 H, t), 2.76 (2 H, t), 2.36 (2 H, t), 2.05 (2 H, m); UV (H_2O) λ_{max} 220, 274, 282 nm (sh).

F5: FABMS (+ ion) m/z (relative intensity) 488 (7, M + H + glycerol), 418 (10, M + Na), 397 (25), 396 (100, M + H), 395 (8), 352 (5), 331 (6), 330 (30); ^1H NMR, see Table I; UV (H_2O) λ_{max} 218, 273, 280 nm (sh).

F3: FABMS (+ ion) m/z (relative intensity) 663 (5), 662 (12, M + Na), 655 (5), 654 (13, M + CH_2 + H), 642 (12), 641 (37), 640 (100, M + H), 596 (5), 595 (5); ^1H NMR (Me_2SO-d_6) δ 8.20 (1 H, d), 8.12 (1 H, d), 7.85 (1 H, t), 6.94 (2 H, d), 6.64 (2 H, d), 4.12 (2 H, m), 3.15 (2 H, m), 2.56 (2 H, t), 2.44 (2 H, m), 2.15 (6–8 H, m), 2.11 (2–3 H, m), 1.95 (3–4 H, m), 1.65 (5–7 H, m); UV (H_2O) λ_{max} (ϵ) pH 2, pH 7, 218 (11 000), 275 (1300), and 282 nm (sh) (1100); λ_{max} (ϵ) pH 12, 237 (11 000) and 291 nm (2400); calcd for $C_{28}H_{37}N_3O_{14}$, M_r 640.2353 (M + H); found, M_r 640.2340 (M + H, HRFABMS).

TCA: FDMS m/z (relative intensity) 301 (23, M + K), 299 (60, M + CH_2 + Na), 286 (33), 285 (100, M + Na), 278 (11), 277 (72, M + CH_2 + H), 264 (27), 263 (100, M + H), 245 (5), 219 (8); ^1H NMR (D_2O) δ 2.74 (1 H), 2.49 (2 H), 1.91 (2 H) (all m).

F1: EIMS (70 eV) m/z (relative intensity) 127 (30, M), 126 (18), 111 (8), 110 (11), 96 (8), 81 (16), 80 (28), 53 (16), 44 (100), 43 (38), 41 (12), 39 (17), 38 (8), 36 (27), 31 (17), 30 (37), 29 (13), 28 (21); HREIMS, 110.0604 (calcd for C_6H_8NO, 110.0606), 80.0501 (calcd for C_5H_6N, 80.0500), 43.0422 (calcd for C_2H_5N, 43.0422); ^1H NMR (Me_2SO-d_6) δ 8.24 (2–3 H, br), 7.52 (1 H, s), 6.45 (1 H, s), 4.99 (1 H, t), 4.23 (2 H, d), 3.98 (2 H, s); ^1H NMR (D_2O + Na_3PO_4; TSP, 0.00) pH 1.3 and 7.2 δ 7.58 (1 H, s), 6.60 (1 H, s), 4.51 (2 H, s), 4.21 (2 H, s); pH 11.5 δ 7.47 (1 H), 6.31 (1 H), 4.49 (2 H), 3.76 (2 H) (all s); ^1H NMR ($CDCl_3$; Me_4Si, 0.00) δ 7.33 (1 H), 6.20 (1 H), 4.52 (2 H), 3.80 (2 H) (all s); UV (H_2O) pH 2 and 7 λ_{max} 214 nm (ϵ estimated 5000–10 000); calcd for $C_6H_9NO_2$, M_r 127.0633; found, M_r 127.0635 (HREIMS).

Alkaline Hydrolysis of Methanofuran. Alkaline hydrolysis of methanofuran (2 mg) was performed in 1 N potassium hydroxide (0.5 mL) at 100 °C for 1 h in an evacuated, sealed glass ampule. The hydrolysate was dried in a vacuum oven, redissolved in water, and purified by HPLC in the same manner as the acidic hydrolysate fragments except that a

(13) Johnson, L. F.; Jankowski, W. C. "Carbon-13 NMR Spectra"; Wiley-Interscience: New York, 1972; Spectra No. 1246 and 251.

(14) Stothers, J. B. "Carbon-13 NMR Spectroscopy"; Academic Press: New York, 1972; p 147.

(15) Leigh, J. A.; Rinehart, K. L., Jr.; Wolfe, R. S. *Biochemistry*, submitted for publication.

(16) The apparent homologues arise from partial conversion of the TCA unit to its methyl esters during purification of methanofuran, which involves methanol.[3]

15-min (60 mL) linear gradient (0-70%) was used. The alkaline hydrolysis fragment ALK 1 was detected by UV absorbance (215 nm, absorbance range 0.5) and eluted at 32 mL after the start of the gradient.

ALK 1: EIMS (70 eV) m/z (relative intensity) 246 (3, M), 217 (8), 200 (9), 110 (13), 94 (26), 82 (20), 81 (4), 80 (6), 44 (32), 43 (30), 38 (20), 36 (58), 32 (74), 31 (100), 29 (51); HREIMS 217.1106 (calcd for $C_{13}H_{15}NO_2$, 217.1103), 200.0861 (calcd for $C_{13}H_{12}O_2$, 200.0837), 110.0598 (calcd for C_6H_8NO, 110.0606), 94.0436 (calcd for C_6H_6O, 94.0419), 82.0635 (calcd for C_5H_8N, 82.0657); ^{1}H NMR (Me_2SO-d_6) δ 8.34 (2 H, br), 7.90 (2 H, br), 7.83 (1 H, s), 7.14 (2 H, d), 6.92 (2 H, d), 6.60 (1 H, s), 4.88 (2 H, s), 4.04 (2 H, br s), 2.94 (2 H, m), 2.76 (2 H, t); ^{1}H NMR (D_2O) δ 7.71 (1 H, s), 7.32 (2 H, d), 7.08 (2 H, d), 6.70 (1 H, s), 5.05 (2 H, s), 4.22 (2 H, s), 3.27 (2 H, t), 2.97 (2 H, t); calcd for $C_{14}H_{18}N_2O_2$, M_r 246.1368; found: M_r 246.1351 (HREIMS).

Resolution of D- and L-Glutamate by GC. Methanofuran (1 mg) was hydrolyzed in 6 N hydrochloric acid at 110 °C for 20 h in an evacuated, sealed tube. The hydrolysate was dried in a vacuum oven in the presence of sodium hydroxide pellets. Trifluoroacetyl methyl esters prepared from the amino acids in the hydrolysate and from standard L-glutamic acid (L-Glu) and D-Glu were analyzed by GC using a capillary column filled with 10% *N*-lauroyl-*N'-tert*-butyl-L-valinamide on 60-80-mesh Chromosorb WAW as described by Pandey et al.[17] The injector temperature was 150 °C, the detector 270 °C, and the carrier gas was helium (1 mL/min) with the column temperature isothermal at 110 °C for 5 min, followed by a gradient (110-140 °C, 5 °C/min), followed by isothermal at 140 °C. The derivatives of D- and L-Glu eluted at 24.3 and 25.3 min, respectively, while the Glu in the hydrolysate comigrated with L-Glu, as it did when co-injected with L-Glu.

Acetyl Derivative of F1. A sample of F1 (1 mg) was treated with acetic anhydride in anhydrous pyridine for 30 min at room temperature and the product was dried under a stream of nitrogen: ^{1}H NMR ($CDCl_3$) δ 7.38 (1 H, s), 6.27 (1 H, s), 5.75 (1 or 2 H, t or m), 4.92 (2 H, s), 4.42 (2 H, d), 2.25 (1 H, s), 2.15 (2 H, s), 2.05 (3 H, s), 2.00 (3 H, s).

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Fluorine Recognition at the Active Site of (*N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl)-α-chymotrypsin

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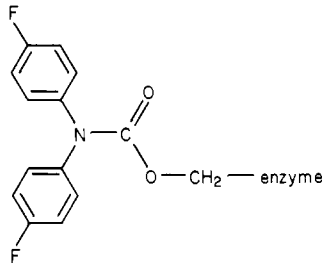
Abstract: *N*-(4-Fluorophenyl)-*N*-phenylcarbamoyl chloride reacts with α-chymotrypsin to give a covalenty modified protein which is devoid of catalytic activity. The fluorophenyl ring of this protein may be found in two magnetically distinct environments; fluorine chemical shift effects suggest that one of these is the substrate specificity pocket at the active site. A competition exists between the fluorophenyl ring and the unsubstituted aromatic ring for residence at each environment, and the NMR observations indicate that the fluorophenyl ring is found preferentially in the active site pocket, a result consistent with the greater hydrophobicity of this group and the nature of the pocket. The kinetics of rotation of the diphenylamino group in both the native and denatured forms of the modified protein have been examined by NMR line shape methods, with results very similar to those obtained with (bis(4-fluorophenyl)carbamoyl)-α-chymotrypsin.

In earlier work we showed that bis(4-fluorophenyl)carbamoyl chloride reacts stoichiometrically with the proteolytic enzyme α-chymotrypsin to give a material with no catalytic activity[1] and indications that this reaction takes place at the active site of the enzyme in a manner completely analogous to the corresponding reaction of diphenylcarbamoyl chloride[2] were described. As structure I suggests, the two aromatic rings of the diphenylamino moiety are magnetically nonequivalent. This nonequivalence is borne out in the fluorine magnetic resonance spectrum of I; two broad signals of equal intensity separated by about 3.4 ppm are observed. One of these signals is at the approximate chemical shift expected for a fluorophenyl ring in solution whereas the other shift is downfield of this position. Derivatives of chymotrypsin which have *p*-fluorophenyl rings able to access the active site pocket usually exhibit large, downfield shifts away from the position observed for the same fluorine resonance in the denatured form of the enzyme derivative.[3-6] Thus, it was suggested that one fluorophenyl ring of modified enzyme I, represented by the low-field signal, resides in the specificity pocket at the active site while the other is placed in a second environment that does not exert as strong an effect on the chemical shift. In the earlier work, various NMR experiments were used to determine the kinetics of diphenylamino group rotation in I; this motion interchanges



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