TAUTOMERIC COMPOSITION OF D-FRUCTOSE PHOSPHATES IN SOLUTION BY FOURIER TRANSFORM CARBON-13 NUCLEAR MAGNETIC RESONANCE

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SUMMARY: The Fourier transform ^{13}C magnetic resonance spectra of D-fructose 6-phosphate (F6P) and D-fructose 1,6-diphosphate (FDP) were obtained. The signal assignments made on the basis of ^{13}C chemical shifts and ^{13}C-^{31}P spin-spin couplings indicate that the earlier assignments of the C-4 and C-5 resonances of α- and β-fructofuranose in oligosaccharides and D-fructose [Allerhand, A. and Doddrell, D., J. Amer. Chem. Soc., 93, 2777, 2779 (1971)] should be reversed. Integration of signal intensities yields the following equilibrium composition at 35°C: F6P, α-anomer 19±2% and β-anomer 81±2%, FDP, α-anomer 23±4% and β-anomer 77±4%. Less than 1.5% keto or hydrated keto form is present in solutions of either fructose phosphate. The bearing of these findings on the tautomeric specificity of phosphofructokinase is discussed.

The phosphate accepting substrate and corresponding product of the key glycolytic enzyme phosphofructokinase (PFK) are D-fructose 6-phosphate (F6P) and D-fructose 1,6-diphosphate (FDP) respectively. Three tautomeric forms are possible for each of these reducing sugar phosphates in solution. These include an α-anomer, a β-anomer and a keto form (Fig. 1). A hydrated keto form (gem diol) can also be envisioned. Since the affinity of PFK toward F6P and FDP will probably vary according to their isomeric form, we deemed it meaningful to determine the tautomeric composition of these two sugar phosphates in solution.

Previous investigators have sought to determine the composition of F6P and FDP in solution through ultraviolet (1-4), optical rotatory dispersion (1, 3), circular dichroic (1, 2), infrared (2, 4), proton NMR (4),

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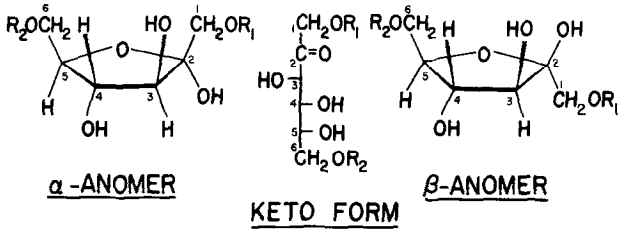

FF :R_1 & R_2 = H
F6P:R_1 = H & R_2 = PO_3^{-2}
FDP:R_1 & R_2 = PO_3^{-2}

Fig. 1. Structures of the tautomeric forms of D-fructofuranose (FF), D-fructose 6-phosphate (F6P) and D-fructose 1,6-diphosphate (FDP).

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^{31}P NMR (4, 5), and continuous wave ^{13}C NMR (CMR) spectroscopy (4, 6). None of these approaches has been conclusive in assessing quantitatively the contribution of all tautomeric forms; consequently, a considerable controversy has arisen (2, 6). With the recent development of the sensitive and rapid pulse Fourier transform technique (7), CMR has emerged as a powerful tool in the determination of the tautomeric composition of reducing sugars in solution (8-11). In an attempt to resolve the existing controversy and in continuation of our studies on the molecular basis of the action of PFK (12-15) we have applied the Fourier transform CMR method to the determination of the tautomeric composition of F6P and FDP in solution.

MATERIALS AND METHODS: Highest quality D-fructose 6-phosphate, disodium salt, and D-fructose 1,6-diphosphate, tetrasodium salt, were purchased from Sigma Chemical Company. These salts were dissolved in D_2O to form 0.70 M solutions which were submitted to CMR analysis. The pD of F6P and FDP solutions were 8.6 and 7.9 respectively. The CMR spectra were obtained on a Varian XL-100 NMR Spectrometer operating at 23.5 KG for ^{13}C acquisition at 25.2 Hz and simultaneous proton noise decoupling at 100 MHz. The utilization of this technique eliminates all ^{13}C-^{1}H spin-spin splitting but retains the coupling patterns caused by ^{13}C-^{31}P interactions. For ultimate sensitivity enhancement, the spectrometer was operated in Fourier transform mode.

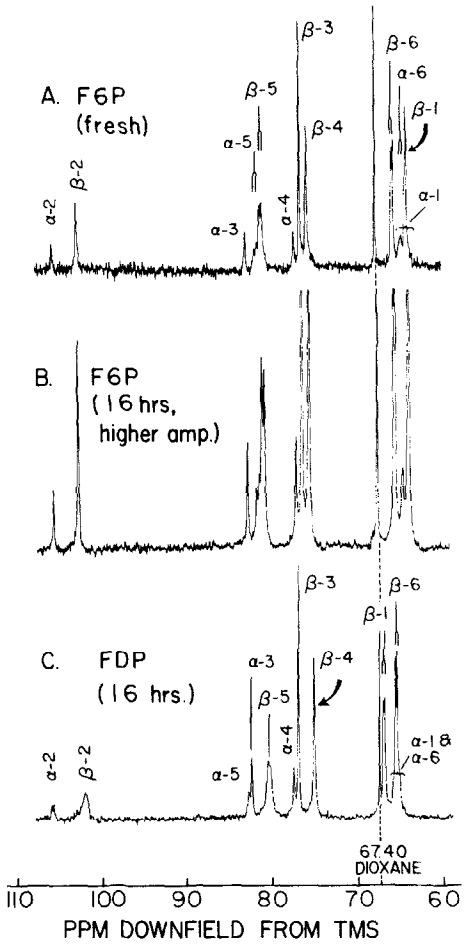

Fig. 2. Natural abundance proton-decoupled CMR spectra obtained in D_2O at 35°C. A, 0.70 M D-fructose 6-phosphate, disodium salt (F6P), pD = 8.6, immediately after dissolution (fresh) and B, at equilibrium (16 hr.) with higher amplification (amp.); and C, 0.70 M D-fructose 1.6-diphosphate, tetrasodium salt (FDP), pD = 7.9, at equilibrium (16 hr.). The three spectra from top to bottom are the result of 1.8×10^3, 19.4×10^3 and 13.6×10^3 accumulations. A slight time dependence is observed in the spectrum of F6P, there being 5% more β-anomer present at equilibrium than immediately after dissolution.

Data were accumulated in a Varian 620i computer employing 2000 Hz sweep width in 4000 points (resolution 0.5 Hz). Chemical shifts (δ) in parts per million (ppm) were determined relative to internal dioxane and converted to the tetramethylsilane (TMS) scale using $\delta_c^{\text{dioxane}} = +67.40$ ppm. Integrations were obtained electronically after the samples had reached equilibrium (16 hr.).

RESULTS AND DISCUSSION: Inspection of Fig. 1 reveals that the best NMR

probe of the tautomeric composition of F6P and FDP is the C-2 carbon. In the different electronic environments of the carbonyl, gem diol, R- and S-hemiketal functional groups (keto form, hydrated keto form, α- and β-anomer respectively), the C-2 of F6P or FDP would appear as four separate and distinct resonances in the range 220-90 δ(TMS) (16). In fact, the Fourier transform CMR spectrum of each fructose phosphate contains only two resonances in this region. These resonances are readily assigned to the C-2 of the α- and β-anomers (Fig. 2) by comparison with the previously reported (10) chemical shifts of the C-2 resonances of α- and β-fructofuranose (Table I).

The fact that the C-2 resonances of only the α- and β-anomers are detected in the spectra of F6P and FDP indicates that the keto form or its hydrate are present in concentrations less than the lower limit of sensitivity of the CMR analytical method (0.01 M). This is equivalent to less than 1.5% when calculated on the basis of the total fructose phosphate concentration present (0.70 M). Absence of appreciable keto form is further substantiated by our observation (17) that the proton NMR spectra of both fructose phosphates at 300 MHz reveal no detectable proton exchange after 16 hr. of sample dissolution in basic D_2O. This indicates that labile protons on carbon atoms adjacent to a carbonyl group (characteristic of the keto form) are not present in solutions of F6P and FDP. Thus it is clear that these fructose phosphates in solution do not exist to an appreciable extent in the keto or hydrated keto form as claimed by Avigad et al. (1) and McGilvery (3).

Having established that our CMR spectra contain resonances due only to α- and β-anomers, specific assignments (Table I and Fig. 2) can be made for F6P and FDP through comparison with the previously assigned resonances of the α- and β-anomers of D-fructofuranose (10). Such a comparison is justified since phosphorylation of a hydroxymethylene carbon deshields it by only a few ppm (18-20). Theoretically, six resonances for each anomer or twelve total ^{13}C resonances for each fructose phosphate should be observed. How-

TABLE I

^{13}C Chemical Shifts and ^{31}P-^{13}C Coupling Constants of D-Fructofuranose (FF),[a] D-Fructose 6-phosphate (F6P)[b] and D-Fructose 1,6-diphosphate (FDP)[b].

Compound and Tautomeric form	C-1	C-2	C-3	C-4	C-5	C-6
α FF[a]	62.1	105.3	83.0	77.0	82.2	62.1
α F6P	c	105.34	82.59	76.93	81.36[e]	64.47[f]
α FDP	d	105.7[g]	82.40	77.47	82.7[g]	d
β FF[a]	63.9	102.4	76.5	75.5	81.5	63.3
β F6P	63.75	102.40	76.24	75.42	80.77[h]	65.38[i]
β FDP	66.88[j]	102.0[g]	76.90	75.09	80.4[g]	65.44[k]

a) Shifts for FF reported in (10). To facilitate comparison these shifts, reported previously in the CS_2 scale ($\delta_c^{dioxane}$ = -126.20 ppm), have been converted to the TMS scale using $\delta_c^{dioxane}$ = +67.40 ppm. The α,β C-4 and α,β C-5 shifts of FF have been reassigned (see text).

b) Chemical shifts are expressed in ppm downfield from TMS. The shifts and coupling constants are estimated to be accurate to ± 1 Hz and ± 0.04 ppm except where noted.

c) Buried in F6P β-1 resonance.

d) Buried in FDP β-6 resonance.

e) Doublet, $^3J_{^{31}POC_6{}^{13}C_5}$ = 8.1 Hz.

f) Doublet, $^2J_{^{31}PO^{13}C_6}$ = 2.8 Hz.

g) Broadened, coupling is undeterminable, accuracy ± 0.1 ppm.

h) Doublet, $^3J_{^{31}POC_6{}^{13}C_5}$ = 7.3 Hz.

i) Doublet, $^2J_{^{31}PO^{13}C_6}$ = 4.2 Hz.

j) Doublet, $^2J_{^{31}PO^{13}C_1}$ = 4.0 Hz.

k) Doublet, $^2J_{^{31}PO^{13}C_6}$ = 4.5 Hz.

ever, resonances of the α-anomer C-1 of F6P and FDP and the α-anomer C-6 of FDP are not apparent due to their overlap with the β-anomer C-1 and C-6 resonances.

The assignments for the C-1 and C-6 methylene resonances of D-fructofuranose in oligosaccharides and D-fructose reported earlier (9, 10) are confirmed by our observation of a $^{31}PO^{13}C$ coupling in the corresponding resonances of F6P and FDP (Table I). This coupling is analogous to that observed in the spectra of nucleotides (18-20). However, it is apparent to us that the earlier assignments (9, 10) of the methine resonances at 82.2 and 81.5 δ(TMS) to the C-4 of the α- and β-anomers of D-fructofuranose respectively are incorrect. Our data indicate that the corresponding resonances in F6P, namely at 81.36 and 80.77 δ(TMS), exhibit a long range $^{31}POC^{13}C$ coupling (Table I), similar to that observed in the CMR spectra of nucleotides (18-20). The only methine ^{13}C nucleus in F6P that can experience such a long range ^{31}P coupling is C-5. Therefore, the assignments for C-4 and C-5 reported for D-fructofuranose in oligosaccharides (9) and D-fructose (10) should be reversed. These corrections in assignments are incorporated in Table I.

After specific assignments have been allocated to the α- and β-anomers of F6P and FDP, the percentage of each tautomer present can be measured through integration. This is feasible quantitatively since Allerhand et al. (19) have shown that all carbons of saccharides have the same nuclear Overhauser enhancement. Comparison of the intensities of the α and β resonances of each carbon in F6P and FDP yields the following equilibrium composition at 35°C: F6P, α-anomer 19±2% and β-anomer 81±2%; FDP, α-anomer 23±4% and β-anomer 77±4%. A greater error is present in the FDP integration due to more overlap and broadening in the spectrum of FDP (Fig. 2 and Table I).

These results are in general agreement with the approximate values for F6P and FDP anomeric composition obtained through continuous wave CMR by Benkovic et al. (6) and through Fourier transform ^{31}P NMR by Gray (5). However, they lend no support to the assumption of the former investigators that F6P exists as much as 5% in the keto form. The use of CMR spectroscopy

in the present study seems to provide more accurate data over the ^{31}P NMR approach used by Gray (5) since the data obtained by the latter method are based on the very closely overlapping C-1 and C-6 phosphate ^{31}P resonances of FDP. Moreover, the Fourier transform technique employed in our CMR approach provides certain experimental advantages over the continuous wave technique employed Benkovic et al. (6). These advantages are: (a) the ability to obtain the entire CMR spectrum of F6P and FDP rather than the anomeric carbon region only, (b) a greater signal-to-noise ratio and consequently a better integration of resonance intensities, and (c) the avoidance of extensive decomposition of samples due to shorter spectrum acquisition time.

The thermodynamic predominance of the β-anomer in solutions of both substrate and product of PFK suggests that this tautomer might be the form of F6P involved in the enzyme-substrate complex. We have obtained further experimental evidence in support of this contention from a study on the structural specificity of rabbit muscle PFK with respect to the phosphate acceptor substrate. The latter findings will be reported elsewhere.

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