

Determination of the Rate-Limiting Steps and Chemical Mechanism of Fructokinase by Isotope Exchange, Isotope Partitioning, and pH Studies[†]

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ABSTRACT: Isotope exchange studies show that beef liver fructokinase has a random kinetic mechanism in which release of fructose from the enzyme is slower than the catalytic reaction. The stickiness of fructose in the presence of MgATP is confirmed by isotope partition studies, which show it to be released 0.53 times as fast as V_i/E_i in the presence, and 80–130 times as fast in the absence of MgATP. Fructose-1-P release from its binary complex is not at all rate limiting in the forward direction since no exchange of MgADP back into MgATP could be observed during the forward reaction. Failure to find any isotope effect by the equilibrium pertur-

bation method with $[1-^{18}\text{O}]$ fructose (upper limit, 1.003) shows that P–O bond cleavage or formation is not rate limiting. The pH profiles for the forward reaction show a group (probably carboxyl with pK 5.7–6.0 and $\Delta H_{\text{ion}} = 0$) that must be ionized and a group (perhaps lysine, with pK 9–10, and ΔH_{ion} 5–9 kcal/mol) which must be protonated for activity. The profile for the back reaction shows only a group with pK 5.5–6 that must be protonated for activity. A chemical mechanism is proposed in which a carboxyl group on the enzyme accepts a proton from the 1-hydroxyl of fructose during the forward reaction and donates it back during the reverse reaction.

In the previous paper (Raushel and Cleland, 1977), we have reported initial velocity, product, and dead-end inhibition studies that show that beef liver fructokinase has a random sequential mechanism in which E·MgATP·Fru-1-P and E·MgADP·fructose dead-end complexes form. In the present paper, we present experimental evidence that the rapid equilibrium assumption does not hold because fructose dissociates slowly from the enzyme in the presence of MgATP. In addition, pH studies are reported which suggest that the chemical mechanism involves a carboxyl group accepting the proton from the 1-OH of fructose during the forward reaction and donating it to the same oxygen in the reverse reaction.

Materials and Methods

Bovine liver fructokinase was isolated as described by Raushel and Cleland (1977). 2,5-Anhydro-D-mannitol-1-P was synthesized enzymatically with yeast hexokinase (Raushel and Cleland, 1973). $[^{14}\text{C}]$ -2,5-anhydro-D-mannitol was synthesized from $[^{14}\text{C}]$ glucosamine by the procedure of Bera et al. (1956). L-Sorbose-1-P was a gift of H. A. Lardy.

Enzymatic Synthesis of D-Tagatose-1-P. D-Tagatose-1-P was prepared by incubating 25 mM D-tagatose, 30 mM ATP, 30 mM MgCl_2 , 25 mM triethanolamine hydrochloride, pH 8.0, 50 mM KCl, and 6 units of fructokinase in a volume of 25 mL for 48 h. The pH was adjusted to 8.0 with dilute NH_3 and the reaction mixture applied to a 0.8×15 cm column of Dowex-1-borate and eluted with a linear gradient of 0.1–0.4 M ammonium tetraborate, pH 8.0. Fractions were assayed for ketoses (Roe et al., 1949) and total phosphate (Bartlett, 1959). Those fractions containing D-tagatose-1-P were pooled and the borate was removed by repeated evaporation with methanol. The yield based on total phosphate was 87% ($[\alpha]^{25}_{\text{D}} - 12^\circ$). A similar procedure was used to prepare D-xylulose-1-P

(yield 66%; $[\alpha]^{25}_{\text{D}} + 3.4^\circ$) and 2,5-anhydro-D-mannose-6-P (yield 58%, $[\alpha]^{25}_{\text{D}} + 2.3^\circ$).

$[^{14}\text{C}]$ -D-Tagatose was made by the epimerization of $[^{14}\text{C}]$ -D-galactose by sodium aluminate (Haack et al., 1964). The yield was 35% as determined by assay with fructokinase.

Preparation of $[1-^{18}\text{O}]$ -D-Sorbitol. One gram of anhydrous D-glucose, 1.0 g of H_2^{18}O (97.4% ^{18}O), and 0.005 mL of concentrated HCl were incubated for 5 days at room temperature to exchange the aldehyde oxygen of glucose (Rittenberg et al., 1961). The glucose was reduced to D-sorbitol by the addition of 0.20 g of solid NaBH_4 over a period of 1 h and then stirred overnight. The solvent was removed under vacuum and the residue dissolved in 100 mL of H_2O . The pH was lowered to 3.5 with IR-120 (H^+), and after filtration the solution was concentrated and the boric acid removed by repeated evaporation with methanol. The yield was 91% by assay with sorbitol dehydrogenase, and the sorbitol had 81% ^{18}O at carbon 1 as determined by mass spectral analysis of its hexaacetate derivative.

Isotope Exchange at Equilibrium. The initial velocity of isotopic exchange at equilibrium was measured as described by Morrison and Cleland (1966). Reaction mixtures of 0.25 or 0.50 mL contained substrates and products at the calculated equilibrium concentrations with a radioactive label in one of the substrates. Each reaction mixture contained 100 mM KCl, 50 mM Pipes¹ (piperazine-*N,N'*-bis(2-ethanesulfonic acid)), 5 mM excess MgCl_2 , and various amounts of substrates and products. The reaction was started either by the addition of fructokinase or the labeled compound. The sugar* \rightarrow sugar-P exchange was measured by spotting aliquots at various times on discs of DEAE-cellulose paper and washing away the unphosphorylated sugar with water. The ATP* \rightarrow ADP exchange was measured by spotting aliquots at various times on sheets of DEAE-cellulose paper and then separating the nu-

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¹ Abbreviations used: Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DEAE, diethylaminoethyl; FruP, fructose 1-phosphate.

cleotides by chromatography with ammonium formate, 0.6 M, pH 3.1.

Nonequilibrium Isotope Exchange. In a volume of 3.0 mL, fructokinase was incubated with 50 mM Pipes (pH 7.0)–100 mM KCl–9.0 mM MgCl₂–3.0 mM ATP–1.5 mM [³H]ADP (6.9 × 10⁵ cpm/μmol)–10.0 mM D-fructose until the D-fructose-1-P concentration was 0.163 mM. The reaction was stopped by the addition of 0.1 mL of 5 N HClO₄ and a drop of CCl₄. The solution was vigorously vortexed and the precipitated protein was removed by centrifugation. The ATP was isolated by chromatography on Dowex-1-Cl and counted. A control contained everything except fructose.

Isotope Partitioning. Isotope partitioning experiments were done according to the procedure of Rose et al. (1974). A 0.2-mL solution of 0.80 unit of fructokinase, 2.0 mM [¹⁴C]-D-fructose (4540 cpm/nmol), 100 mM KCl, 50 mM Pipes, and 5 mM MgCl₂ was rapidly added with a Gilson automatic pipet to a vigorously stirred 5.0-mL solution of 0.4 M unlabeled D-fructose, 5 mM excess MgCl₂, 50 mM Pipes, 100 mM KCl, and varying levels of MgATP. After 2 s the fructokinase was denatured by the addition of 0.20 mL of 5 N HClO₄ and a few drops of CCl₄. The solution was vigorously vortexed, centrifuged, and the pH adjusted to 8.0 with KOH. The [¹⁴C]-D-fructose-1-P was isolated by chromatography on Dowex-1-Cl. A blank was run with 10 mM MgATP in which the [¹⁴C]-fructose was present in the 5.0-mL solution and not in the 0.2-mL one. This blank (0.154 nmol) was then subtracted from all experimental values to correct for radioactive impurities and any [¹⁴C]fructose-1-P formed from the diluted fructose during the 2-s incubation. The resulting net values ranged from 0.023 to 0.40 nmol.

Equilibrium Perturbation. In a volume of 3.0 mL, 10 units of sorbitol dehydrogenase were incubated with 50 mM Pipes, pH 7.0, 100 mM KCl, 1.87 mM NAD, 2.00 mM [1-¹⁸O]-D-sorbitol, 9.00 mM ADP, 16.0 mM MgCl₂, 9.16 mM fructose-1-P, and levels of ATP near 1 mM until the reaction came to equilibrium as indicated by no further change in absorbance at 340 nm. The concentrations of [1-¹⁸O]fructose and NADH produced were 0.170 mM. One unit of fructokinase was then added and the absorbance at 340 nm was monitored. The experiment was repeated with different levels of ATP until a level was found (1.01 mM) which produced no change in absorbance at 340 nm with time after fructokinase addition.

pH Studies. Fructokinase activity in the forward and reverse direction was measured spectrophotometrically as described by Raushel and Cleland (1977). The buffers for the pH profile of the forward reaction were 25 mM each in acetate, cacodylate, diethyl malonate, and *p*-phenolsulfonate titrated to the desired pH by the addition of KOH. For the pH profile of the back reaction, the buffers were 50 mM each in acetate and cacodylate titrated to the desired pH by the addition of KOH.

Data Processing. Data from single reciprocal plots were fitted to eq 1. The pH profiles were fitted to either eq 2 or 3 and Arrhenius plots were fitted to eq 4.

$$v = \frac{VA}{K + A} \quad (1)$$

$$\log Y = \log \left(\frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \right) \quad (2)$$

$$\log Y = \log \left(\frac{C}{1 + \frac{K_2}{H}} \right) \quad (3)$$

TABLE I: Equilibrium Constants for Fructokinase.^a

Substrate	K _{eq}
D-Fructose	460 (330) ^b
L-Sorbose	140
D-Tagatose	220
D-Xylulose	410
2,5-Anhydro-D-mannitol ^c	1060
2,5-Anhydro-D-mannose ^c	850

^a pH 7.0, 100 mM KCl, 25 °C. ^b Calculated on the assumption that the β-furanose anomers of D-fructose (21%) (Angyal and Bethell, 1976) and D-fructose-1-P (15%) (Benkovic et al., 1973) are the reactants. ^c In order to conserve the enzyme, the equilibrium constants for these substrates were determined with yeast hexokinase since fructokinase and hexokinase catalyze the same reaction for these substrates.

$$Y = a \left(\frac{1}{T} \right) + b \quad (4)$$

In eq 1, *V* is the maximum velocity and *K* is the apparent Michaelis constant of *A*. In eq 2 and 3, *K*₁ and *K*₂ are acid dissociation constants for groups that must be deprotonated and protonated, respectively, for activity, *H* is the hydrogen ion concentration, and *C* is the value of *Y* attained at the optimum state of protonation. In eq 4, *Y* is the p*K*, *T* is the absolute temperature (K), *a* is Δ*H*_{ion}/2.303*R*, and *b* is a constant. Experimental data were fitted to the appropriate equation by the least-squares method assuming equal variances for *V*, log *Y*, or *Y* (Wilkinson, 1961) and using the Fortran programs of Cleland (1967).

Results

Equilibrium Constants. Equilibrium constants for the several substrates of fructokinase were determined at pH 7.0 by making up reaction mixtures that were close to equilibrium and varying MgATP to produce product-to-reactant ratios that bracketed *K*_{eq}. Enzyme was added and after 2-h incubation the fructokinase was denatured by vortexing with a few drops of CCl₄. The final MgATP concentration was determined by an enzymatic assay with yeast hexokinase and glucose-6-phosphate dehydrogenase. The change in the MgATP concentration was then plotted against the initial product-to-reactant ratio, with *K*_{eq} being taken as the point where the resulting curve was 0 on the ΔMgATP axis. Equilibrium constants, defined as:

$$\frac{[\text{sugar-P}][\text{MgADP}]}{[\text{sugar}][\text{MgATP}]}$$

at pH 7.0 and 25 °C are listed in Table I. A corrected equilibrium constant for D-fructose is also shown in which only β-D-fructofuranose and β-D-fructofuranose-1-P are assumed to be the reactants.

Equilibrium Isotopic Exchange. The initial velocity of isotopic exchange was measured by determining the rate in which [¹⁴C]-D-fructose was incorporated into D-fructose-1-P and the rate in which [¹⁴C]ATP was incorporated into ADP at chemical equilibrium. Shown in Figure 1 is the effect of varying the fructose–fructose-1-P pair in constant ratio while holding the ATP–ADP pair constant. Both exchanges give linear reciprocal plots, but the ATP* → ADP exchange is 3.6 times faster than the fructose* → fructose-1-P exchange at the point of maximal exchange. Also shown in Figure 1 is the effect of varying the ATP–ADP pair in constant ratio. Both ex-

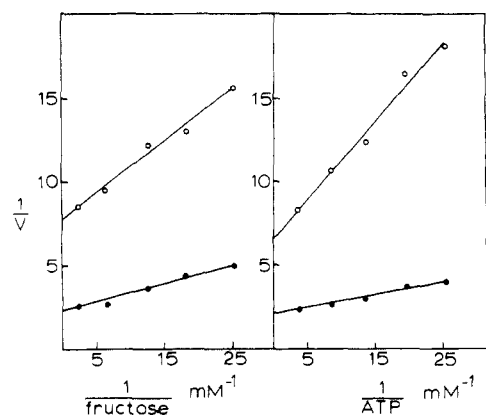


FIGURE 1: (Left) Isotope exchange with fructose and fructose-1-P as the varied reactants. Fructose-1-P/fructose = 30; MgATP, 0.28 mM; MgADP, 4.2 mM; 0.061 unit of fructokinase. (Right) Isotope exchange with MgATP and MgADP as varied reactants. MgADP/MgATP = 15; fructose, 0.40 mM; fructose-1-P, 12.0 mM; 0.061 unit of fructokinase. Data fitted to eq 1. Velocities are expressed as nmol min^{-1} (unit of enzyme) $^{-1}$. (Open circles) fructose* \rightarrow fructose-1-P exchange; (closed circles) ATP* \rightarrow ADP exchange.

changes again give linear plots and the maximum ATP* \rightarrow ADP exchange is 3.2 times faster than the maximum fructose* \rightarrow fructose-1-P exchange.

Isotopic exchange rates at one level of reactants and products for L-sorbose, D-tagatose, and 2,5-anhydro-D-mannitol are presented in Table II along with the maximal exchange rates for D-fructose from Figure 1.

Isotopic Exchange from ADP into ATP during the Forward Reaction. With saturating levels of reactants, exchange of MgADP into MgATP did not occur during the forward reaction under conditions where MgADP inhibited the reaction rate by 50%. In the control with no fructose, 11 980 cpm was recovered in the ATP (presumably a contaminant in the labeled ADP), while with fructose, 11 570 cpm were recovered.

Isotope Partitioning. A binary complex of fructokinase and [^{14}C]fructose was diluted into 25 volumes of a solution containing excess unlabeled fructose and variable levels of MgATP and reaction was terminated after 2 s. The number of nmol of radioactive D-fructose-1-P isolated was plotted vs. the concentration of MgATP and the data were fitted to eq 1. The amount of radioactive D-fructose-1-P formed at infinite MgATP (P_{max}), was 0.97 ± 0.09 nmol and the apparent Michaelis constant (K') for MgATP from this plot was 14 ± 2 mM.

The same type of isotope partitioning experiments were also carried out with [$\gamma\text{-}^{32}\text{P}$]ATP and eight times as much enzyme. However, no trapping of [^{32}P]D-fructose-1-P could be detected even at 500 times the K_m of D-fructose.

Attempted Measurement of ^{18}O Isotope Effect by Equilibrium Perturbation. No ^{18}O isotope effect could be detected with fructokinase using [$1\text{-}^{18}\text{O}$]fructose and the equilibrium perturbation technique of Schimerlik et al. (1975). Since the fructokinase reaction cannot be followed spectrophotometrically, the reaction was coupled to sorbitol dehydrogenase. When fructokinase was added to a solution of reactants and products that was at chemical equilibrium with respect to sorbitol dehydrogenase and fructokinase, but had oxygen-18 at carbon 1 of sorbitol and fructose, no change in the NADH concentration could be detected during a period of at least 1 h. Assuming that a change in the NADH concentration of 0.4 μM could have been detected (this corresponds to a pertur-

TABLE II: Isotopic Exchange Rates at Equilibrium.

Substrate	Initial velocity of isotopic exchange (nmol min^{-1} (unit of fructokinase) $^{-1}$)		
	Sugar* \rightarrow sugar-P	ATP* \rightarrow ADP	Nucleotide exchange/ sugar exchange
D-Fructose ^a	2.1	7.7	3.6
	2.5	7.9	3.2
L-Sorbose ^b	0.48	1.27	2.6
D-Tagatose ^c	1.8	1.8	1.0
2,5-Anhydro-D-mannitol ^d	0.81	1.9	2.3

^a For conditions, see Figure 1. ^b ATP, 0.20 mM, 3.0 mM ADP, 8.2 mM MgCl_2 , 0.13 mM L-sorbose, 1.23 mM L-sorbose-1-P, 0.29 unit of fructokinase. ^c ATP, 0.20 mM, 3.0 mM ADP, 8.2 mM MgCl_2 , 0.50 mM D-tagatose, 7.2 mM D-tagatose-1-P, and 0.49 unit of fructokinase. ^d ATP, 0.20 mM, 5.3 mM ADP, 10.5 mM MgCl_2 , 0.50 mM 2,5-anhydromannitol, 20 mM 2,5-anhydromannitol-1-P, and 0.12 unit of fructokinase.

bation of 0.89 μM fructose), the ^{18}O isotope effect for fructokinase is less than 1.003, using the calculations of Schimerlik et al. (1975).

Variation of Kinetic Parameters with pH. The pH profile of the forward reaction of fructokinase was determined with 2,5-anhydro-D-mannitol as the varied substrate by varying the 2,5-anhydro-D-mannitol concentration at a constant saturating level of MgATP (3.0 mM ATP and 6 mM MgCl_2). 2,5-Anhydro-D-mannitol was chosen to eliminate any possible effects due to changes in anomeric composition with pH or particularly with temperature (D-fructose is known to change its specific rotation and its anomeric distribution with changes in temperature, concentration, and addition of organic solvents (Tsuzuki et al., 1950; Isbell and Pigman, 1969; Angyal and Bethell, 1976).) In preliminary experiments the Michaelis constant for MgATP was shown to be essentially constant (about 0.25 mM) over the pH range 5.2–9.7 ((2,5-anhydro-D-mannitol) = 15 mM). The pH profiles of V_1 and $V_1/K_{2,5\text{-anhydro-D-mannitol}}$ for fructokinase are plateaus over the pH range 7–9 with activity falling off at high and low pH. The pKs from fits of the data to eq 2 at 15, 25, and 35 $^{\circ}\text{C}$ are shown in Table III. Enthalpies of ionization were calculated from Arrhenius plots of pK vs. $1/T$. From fits to eq 4, enthalpies of ionization of 0 were obtained for the low pH pKs, and 9400 and 4700 cal/mol for the high pH V and V/K pKs.

When the pH profile of the forward reaction was determined with D-fructose as the varied substrate (ATP, 3 mM; MgCl_2 , 6 mM), a very similar profile was found, suggesting that the anomeric composition does not change appreciably with pH at constant temperature. Since fructose was found to give nonlinear double-reciprocal plots (apparent substrate activation) at high concentrations of D-fructose at the lower pH values, the V_1/K_{fructose} profile was obtained from the data in the linear portion of the double-reciprocal plot and the V_1 profile was obtained at a constant level of 25 mM fructose. The profiles are shown in Figure 2, and the pK values from fits to eq 2 are shown in Table III.

The pH profiles of the back reaction were obtained by varying the D-fructose-1-P concentration at a constant level of 2.0 mM ADP and 7 mM MgCl_2 . The $V_2/K_{\text{Fructose-1-P}}$ profile was corrected by assuming that only the dianion of D-fructose-1-P is a substrate and that the second ionization of D-fructose-1-P has a pK of 6.0. The V_2 and the corrected $V_2/$

TABLE III: pK Values from V and V/K Profiles.

Substrate	Apparent pK values at specified temperature					
	15 °C		25 °C		35 °C	
2,5-Anhydromannitol						
V	5.70 ± 0.04	9.66 ± 0.04	5.82 ± 0.04	9.41 ± 0.03	5.72 ± 0.04	9.19 ± 0.04
V/K	6.17 ± 0.03	9.82 ± 0.05	6.12 ± 0.02	9.80 ± 0.05	6.17 ± 0.05	9.58 ± 0.05
Fructose						
V			5.45 ± 0.04	9.63 ± 0.05		
V/K			5.87 ± 0.03	9.99 ± 0.08		
Fructose-1-P						
V			6.34 ± 0.02			
V/K			5.52 ± 0.13			

$K_{\text{Fru-1-P}}$ profiles are shown in Figure 2 and the pKs from fits of the data to eq 3 appear in Table III.

Discussion

Equilibrium Constants. The equilibrium constants for fructokinase with the keto sugars at pH 7, 25 °C, range from 140 to 460. Differences in the anomeric distribution of the phosphorylated and unphosphorylated sugars account for part of the variations seen. For example, when correction is made for the amount of β -D-fructofuranose and β -D-fructofuranose-1-P in solution, the equilibrium constant changes from 460 to 330. The anomeric compositions for the other phosphorylated sugars are not known, so similar corrections cannot be made for these compounds. The corrected equilibrium constant of 330 for fructose is very close to the value of 311 calculated for phosphofructokinase at pH 7, 25 °C, using a ΔG° of -3.40 kcal (Lehninger, 1970). It is clear that the presence of the anomeric hydroxyl on carbon 2 increases the free energy of hydrolysis of the 1-phosphate since the anhydro sugar alcohols give higher K_{eq} values that are closer to the expected value for a sugar phosphate (for example, $K_{\text{eq}} = 1300$ at pH 7 for the hexokinase reaction (Robbins and Boyer, 1957)).

Rate-Limiting Steps. The isotope exchange studies with fructokinase confirm the random kinetic mechanism postulated in the previous paper (Raushel and Cleland, 1977). If the kinetic mechanism were ordered, one of the exchange reactions in Figure 1 would show substrate inhibition. However, the mechanism cannot be described as rapid equilibrium random because with fructose as a substrate the maximum $\text{ATP}^* \rightarrow \text{ADP}$ exchange is 3.2–3.6 times faster than the maximum $\text{fructose}^* \rightarrow \text{fructose-1-P}$ exchange. If catalysis were rate limiting (as it is in a rapid equilibrium random mechanism), the exchange rates would be identical since they would then be limited by the same step. Thus, catalysis cannot be solely rate determining, and the release of fructose and/or fructose-1-P from the enzyme must be at least partly rate limiting for these exchange reactions.

With L-sorbose and 2,5-anhydro-D-mannitol, the nucleotide exchange is also faster than the $\text{sugar}^* \rightarrow \text{sugar-1-P}$ exchange, although the ratios are smaller than with D-fructose. This suggests that these substrates are less sticky than is fructose with fructokinase, or catalysis is more rate limiting. With D-tagatose, however, the exchange rates are identical, which suggests that the rapid equilibrium assumption may be valid for this substrate.

The isotope partitioning technique of Rose et al. (1974) is an excellent method for determining the relative rates of release

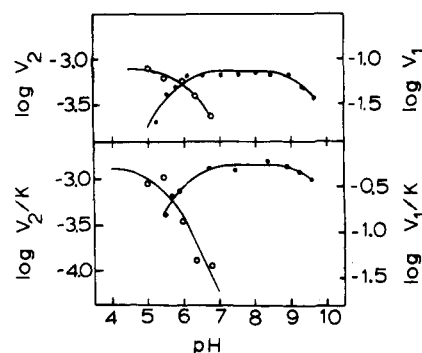


FIGURE 2: (Top) (Closed circles) pH profile of V_1 with fructose as the substrate. Data were fitted to eq 2. (Open circles) pH profile of V_2 with fructose-1-P as substrate. Data fitted to eq 3. (Bottom) (Closed circles) pH profile of V_1/K_{fructose} . Data fitted to eq 2. (Open circles) pH profile of $V_2/K_{\text{Fru-1-P}}$. The dianion of fructose-1-P was assumed to be the active species. Data fitted to eq 3.

of substrates from their complexes with enzyme. In the isotope partitioning experiment with fructokinase, 0.97 ± 0.09 nmol of radioactive fructose-1-P was formed at infinite MgATP using 0.80 unit of enzyme. Using a molecular weight of 56 000 and a specific activity of 17 U/mg for pure enzyme (Raushel and Cleland, 1977) and assuming that both subunits are active, the concentration of enzyme sites is 1.7 nmol. The rate of dissociation of fructose from the E-fructose-MgATP complex (k_7) relative to V_1/E_t is given by:²

$$\frac{k_7}{V_1/E_t} = \left(\frac{E_t/(1 + K_i(\text{fructose})/(\text{fructose}))}{P_{\text{max}}^*} \right) - 1 \quad (5)$$

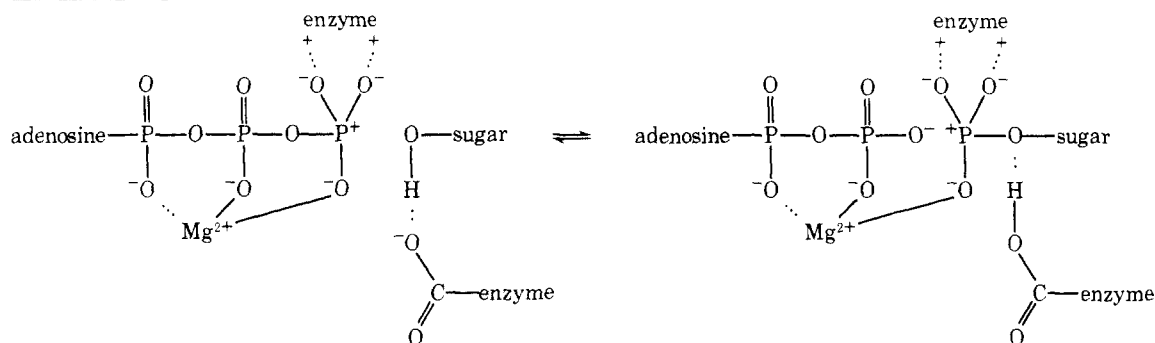
$K_i(\text{fructose})$ is 0.29 mM (Raushel and Cleland, 1977), fructose was 2.0 mM, and thus $k_7/(V_1/E_t) = 0.53$. The rate of dissociation of fructose from the E-fructose complex (k_2) is given by:

$$\left(\frac{K'}{K_{\text{ATP}}} \right) \left(\frac{E_t/(1 + K_i(\text{fructose})/(\text{fructose}))}{P_{\text{max}}^*} \right) \geq \frac{k_2}{V_1/E_t} \geq \frac{K'}{K_{\text{ATP}}} \quad (6)$$

² To be rigorous, what is calculated in eq 5 is the ratio of k_7 to the net rate constant for catalysis and release of the first product (that is, for steps up to and including the first irreversible one). Thus, if V_1/E_t were partly or totally limited by release of the second product, $k_7/(V_1/E_t)$ would be larger than calculated by eq 5. This is unlikely in the present case, since E-fructose-1-P does not appear to be present at appreciable levels in the steady state. These considerations do not apply to eq 6, which is valid under all circumstances (it is really a comparison of k_2/K' with $V_1/K_{\text{ATP}}E_t$, and the latter is unaffected by steps after the first irreversible one).

where K' is the apparent Michaelis constant for MgATP for the isotope partitioning experiment and K_{ATP} is the Michaelis constant for MgATP from initial velocity studies. Using 14 mM for K' and 0.17 mM for K_{ATP} , k_2 is 80–130 times faster than V_1/E_1 .

Thus, fructose is relatively sticky in the ternary complex, but not very sticky at all in the binary complex. No conversion of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into fructose-1-P could be demonstrated, showing that MgATP dissociates from both the binary and ternary enzyme complexes much faster than V_1/E_1 . These results are consistent with the isotopic exchange experiments



which indicated that fructose was sticky while the nucleotides were not.

The equilibrium perturbation method for measuring isotope effects is sensitive enough to use with heavy isotopes such as ^{13}C and ^{15}N (Schimerlik et al., 1975). However, no perturbation was observed with $[1\text{-}^{18}\text{O}]\text{fructose}$, and thus the oxygen-18 isotope effect with fructokinase is less than 1.003. The only oxygen-18 isotope effects previously reported for an enzymatic reaction are for chymotrypsin where values of 1.006–1.018 have been observed (Sawyer and Kirsch, 1975; O'Leary and Marlier, 1976). The absence of an oxygen-18 isotope effect for fructokinase suggests that phosphorus-oxygen bond breaking or making is not rate determining to any extent. However, the equilibrium perturbation technique is largely a measure of the effect on V/K , and a large commitment to catalysis (caused by the stickiness of fructose in the ternary complex) would diminish the apparent isotope effect even if the phosphoryl transfer were partly rate limiting (Northrop, 1975).

An attempt was made to measure $\text{MgADP}^* \rightarrow \text{MgATP}$ exchange during the course of the forward reaction to determine if E-fructose-1-P accumulated in the steady state to any extent. No exchange was observable, and thus it appears that no appreciable amount of E-fructose-1-P is present in the steady state, and fructose-1-P release is not at all rate limiting for the forward reaction, nor probably also for fructose-fructose-1-P exchange.

pH Profiles and the Chemical Mechanism. The V_{\max} profiles for both D-fructose and 2,5-anhydro-D-mannitol are plateaus with activity falling off at both high and low pH. The pK at high pH, which represents a group that must be protonated for activity, is in the range expected for an ϵ -amino group of lysine. The ΔH_{ion} is 9400 cal/mol for the V_{\max} profile, which is close to the value of 10 000–13 000 cal/mol reported by Edsall (1943) for lysine side chains. The function of this group is unknown. It might be simply important for the correct structure of the enzyme, or it might be one of the groups binding the equatorial oxygens of the transferred phosphate so that the phosphorus atom has sufficient positive charge to permit attack by the sugar.³

The pK at low pH represents a group that must be unprotonated for activity of the forward reaction and protonated for activity of the back reaction. The pK is in the range for either a carboxyl or an imidazole residue, but the ΔH_{ion} of 0 is indicative of a carboxyl (Edsall, 1943). The crossover in the pH profiles suggests that this carboxyl is acting to accept the proton from the 1-OH of fructose during catalysis of the forward reaction and to donate a proton to fructose-1-P during catalysis of the back reaction. These results thus suggest that the chemical reaction occurring during phosphate transfer may be represented as:

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³ One of the groups binding the equatorial oxygens on the transferred phosphate may be arginine since the enzyme is slowly inactivated by 2,3-butanedione in borate buffer. Some protection against inactivation is provided by MgATP or fructose, and a greater degree of protection by fructose and MgADP together (Rauschel, 1976).

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Purification and Properties of γ -Butyrobetaine Hydroxylase from *Pseudomonas* sp AK 1[†]

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ABSTRACT: γ -Butyrobetaine hydroxylase (4-trimethylaminobutyrate, 2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) has been isolated from *Pseudomonas* sp AK 1 by ion-exchange, adsorption, and molecular-sieving chromatography. The preparation was homogeneous as judged from electrophoresis in agarose and polyacrylamide gels, isoelectric focusing, and equilibrium sedimentation. The molecular mass was 95 kdaltons as determined by sedimentation equilibrium centrifugation. From electrophoresis in polyacrylamide gel the molecular mass was estimated to 92 kdaltons, from gel filtration through columns of Sephadex G-200 to 86 kdaltons, and from gel filtration through thin layers of Sephadex G-150 and G-200 to 82 kdaltons. Calculation of molecular mass from Stokes radius, sedimentation coefficient, and partial specific volume gave a value of 96 kdaltons, and from the sedimentation coefficient, 93 kdaltons. Gel filtration through Sephadex G-200 in 6 M guanidinium chloride and electrophoresis in polyacrylamide gel containing 3.5 mM sodium dodecyl sulfate resulted in single bands with mobilities corresponding to molecular masses of 39 and 37

kdaltons, respectively, indicating that the enzyme is composed of two polypeptide chains with similar size. NH₂-terminal amino acid sequencing in three cycles resulted in two amino acids in each cycle (Ala + Asn, Ala + Ile, Ala + Ile). The Stokes radius was 3.8 nm, corresponding to a diffusion coefficient of 5.7×10^{-7} cm²/s. A sedimentation coefficient of 5.8×10^{-13} s and a frictional ratio of 1.26 was found. The partial specific volume was 0.729 mL/g at 20 °C as calculated from amino acid analysis. The isoelectric point was 5.1, as determined by isoelectric focusing analysis. The light absorption in the ultraviolet and visible regions was that of a protein without light-absorbing prosthetic groups. The absorption coefficient at 280 nm of a 1.0% solution at pH 6.5 was 12.6. Amino acid analysis by ion-exchange chromatography showed a half-cystine content of 19 mol per 95 kg of protein (23 residues/1000). Thirteen sulfhydryl groups were found by colorimetric analysis before as well as after reduction with NaBH₄, indicating absence of disulfide bonds. Less than 0.1 mol of iron was found per mol of enzyme.

The hydroxylation of γ -butyrobetaine to carnitine was first studied in crude preparations from rat liver (Lindstedt and Lindstedt, 1962, 1970; Lindstedt, 1967a,b). A *Pseudomonas* strain which had been isolated by enrichment culture in γ -butyrobetaine-containing media was then used as the source of γ -butyrobetaine hydroxylase (4-trimethylaminobutyrate, 2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) (Lindstedt et al., 1967, 1970a,b). With crude preparations of this enzyme, it could be demonstrated that 2-oxoglutarate is decarboxylated in stoichiometric amounts with the formation of carnitine, that succinic semialdehyde is not an intermediate in succinate formation, and that molecular oxygen is incorporated into succinate (Lindstedt et al., 1968; Holme et al., 1968; Lindblad et al., 1969). This novel type of oxygenase reaction was then demonstrated for two other enzymes, i.e., prolyl hydroxylase (EC 1.14.11.2), and thymine 7-hydroxylase (EC 1.14.11.6) (for a review, see Hayaishi et al., 1975). There is evidence that thymidine 2'-hydroxylase and lysyl hydroxylase catalyze the same type of reaction, although it has not so far been demonstrated that molecular oxygen is incorporated into succinate formed during the reaction. An-

other 2-oxoacid-dependent hydroxylation—the formation of homogentisate from 4-hydroxyphenylpyruvate—probably occurs with a similar mechanism (Goodwin and Witkop, 1957; Lindblad et al., 1970).

Prolyl hydroxylase has been isolated from various animal sources by several groups (for a review, see Hayaishi et al., 1975). 4-Hydroxyphenylpyruvate dioxygenase has been isolated from human liver (Lindblad et al., 1970; to be published) and from a *Pseudomonas* strain (Lindstedt et al., to be published) as well as from several animal species (Fellman et al., 1972; Nakai et al., 1975).

We now report the isolation and characterization of γ -butyrobetaine hydroxylase from *Pseudomonas* sp AK 1.

Experimental Procedures

Materials. Compounds were obtained from the following sources: γ -butyrobetaine chloride from E. Merck AG, Darmstadt, West Germany; human transferrin from Kabi AB, Stockholm, Sweden; polyamide thin-layer plates from Cheng Chin Trading Co., Ltd., Tapei, Taiwan; BDC-OH¹ and

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¹ Abbreviations used are: Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid); BDC-OH, bis[4-(dimethylamino)phenyl]carbinol; Pth, phenylthiohydantoin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.