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Aldehyde-Induced Adenosine Triphosphatase Activities of Fructose 6-Phosphate and Fructose Kinases†

Ronald E. Viola‡ and W. W. Cleland*

ABSTRACT: Chitose-6-P (2,5-anhydromannose-6-P) induces ATPase activity of fructose-6-P kinase with a V_{max} 2–3% that of the normal kinase reaction with fructose-6-P or 2,5-anhydromannitol. Chitose (and presumably also chitose-6-P) is 52% hydrated in water while chitose deuterated at C-1 is 60% hydrated because of the equilibrium isotope effect of 0.73 on aldehyde hydration. Deuterated chitose-6-P gave a normal isotope effect on V/K of 1.23, but no effect on V_{max}, showing that the free aldehyde is the activator and the hydrated form does not bind appreciably. With fructokinase, chitose can act either as a substrate, being phosphorylated at C-6 when adsorbed with C-6 next to MgATP, or as an inducer of ATPase activity when adsorbed with C-1 next to MgATP. The ATPase has a rate about 25% that of the kinase.

Kinases are enzymes which transfer the γ-phosphate of MgATP to various acceptors. While considerable information about the stereochemistry (Knowles, 1980) and the coordination of Mg to the phosphates of ATP and the products during the reaction (Cleland & Mildvan, 1979) is now available, the chemical mechanism is still not well understood. The ATPase activity seen in the presence of aldehydes corresponding to alcohols which are substrates for certain kinases is thus of considerable mechanistic interest. Such activity was first seen with glycerokinase, which was found by Janson & Cleland (1974) to phosphorylate L-glyceraldehyde with a V_{max} 150% that of glycerol but in the presence of D-glyceraldehyde to show ATPase activity with a V_{max} 30% that of glycerol. We have now found that both fructose-6-P kinase and fructokinase show ATPase activity in the presence of aldehyde analogues of their substrates.

When such activity was first observed, it was thought to result from phosphorylation of the hydrated form of the aldehyde to produce an unstable compound which decomposed nonenzymatically. This hypothesis is readily testable by measuring the deuterium isotope effects on the activation parameters of the aldehyde, since there is a 37% deuterium isotope effect on the hydration equilibrium (Hill & Milosevich, 1976; Lewis & Wolfenden, 1977). For example, an aldehyde that is half-hydrated in water will contain 58% hydrate and 42% free aldehyde when deuterated at C-1. The isotope effect on V/K will either be inverse ($^{D}(V/K) = 0.86$)[1] if the hydrate is the activator or normal ($^{D}(V/K) = 1.16$) if the free aldehyde is the activator. If only the active form can combine with the enzyme, no isotope effect on V_{max} is predicted ($^{D}V = 1.0$). However, an apparent isotope effect on V_{max} will be seen if both the aldehyde and its hydrate combine with the enzyme, but only one is active, because the ratio of the two forms will be different for deuterated and unlabeled aldehyde and the presence of a competitive inhibitor in constant ratio to the variable substrate reduces V_{max} (but has no effect on V/K). As a second example, when an aldehyde is nearly fully hydrated (96% in the case of glyceraldehyde (Swenson & Barker, 1971)), the isotope effect on V/K will either be near unity ($^{D}(V/K) = 0.99$) if the hydrate is the activator or nearly fully expressed ($^{D}(V/K) = 1.35$) if the aldehyde is the activator.

Bar-Tana & Cleland (1974) reported that 2,5-anhydromannitol is a substrate for fructose-6-P kinase and is phosphorylated slightly faster than fructose-6-P. We now report that its aldehyde analogue, 2,5-anhydromannose-6-P (chitose-6-P), induces ATPase activity by fructose-6-P kinase. Through the use of the deuterium isotope effect on aldehyde hydration, we will show that the free aldehyde of chitose-6-P

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‡ Present address: Department of Chemistry, Southern Illinois University, Edwardsville, IL 62026.

[1] ^{D}X is the deuterium isotope effect on X (that is, the ratio of X values for unlabeled and labeled molecules).

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is the activator and that the hydrate does not bind.

Materials and Methods

Fructokinase was prepared from bovine liver by the method of Raushel & Cleland (1977). Buffers were from Calbiochem or Sigma, and NAD was from Boehringer. Dowex resins were from Bio-Rad. Dowex-50 was left in the H^+ form, while Dowex-3 was converted to the free-base form by washing with 0.5 N NaOH. D_2 gas (>99.5 atom %) was from Matheson and $Pd/BaSO_4$ catalyst was from Aldrich. All other enzymes and substrates were from Sigma except as noted below.

Purification of Fructose-6-phosphate Kinase. Fructose-6-phosphate kinase was purified from contaminating ATP by the method of Bock & Frieden (1974) except that the enzyme was dialyzed for about 1 h prior to charcoal treatment to remove turbidity and the dialysis time after charcoal treatment was decreased to 2 h to minimize irreversible denaturation.

Kinetic Assays. Kinetic studies were run in 3.0-mL total volume in 1-cm cuvettes by measuring the absorbance changes at 340 nm. The ATPase reactions were followed by measuring the production of ADP using a pyruvate kinase–lactate dehydrogenase enzyme couple. Assay mixtures for following the ATPase activity of fructokinase contained 100 mM Hepes,[2] pH 8.0, 100 mM KCl, 0.4 mM PEP, 0.4 mM NADH, 0.25 mg each of pyruvate kinase and lactate dehydrogenase, and varying levels of chitose and ATP, with Mg^{2+} present at 1 mM excess over ATP. The reaction was started with 0.1 unit of fructokinase. Assays for the ATPase activity of fructose-6-P kinase contained, in addition to the components above, 10 units of yeast hexokinase to convert chitose to chitose 6-phosphate. The reaction was then started by addition of 10–20 units of fructose-6-phosphate kinase.

The coupled assay for following the production of inorganic phosphate contained the following: 100 mM Hepes (pH 8.0), 1 mM ATP, 5 mM magnesium(II) chloride, 1 mM dihydroxyacetone phosphate, 0.5 mM NAD, 0.1 mM chitose, 5 units of glyceraldehyde-3-phosphate dehydrogenase, 12 units of 3-phosphoglycerate kinase, 20 units of triose phosphate isomerase, and 20 units of hexokinase. It was necessary to remove trace amounts of phosphate from the coupling enzymes by dialysis before the assay mixture was prepared. After chitose had been phosphorylated to chitose-6-P by hexokinase (thus generating sufficient MgADP to react with the diphosphoglycerate produced in the coupled assay) and dihydroxyacetone-P had been equilibrated with glyceraldehyde-3-P by triose-P isomerase, the reaction was started by addition of 10–20 units of fructose-6-P kinase which had been dialyzed to remove phosphate.

The production of inorganic phosphate was also determined by a modification of the phosphomolybdate assay of Lowry & Lopez (1946). After reaction mixtures containing the coupling enzymes needed to determine ADP production had gone to completion, 100 μL of 60% perchloric acid was added to 3 mL of reaction mixture to denature the enzyme. The mixture was vortexed and filtered to remove denatured enzyme. The pH was adjusted to 4 with 1 N sodium acetate (about 0.75 mL), 0.5 mL of ascorbate (1 g in 100 mL of water) and 0.5 mL of ammonium molybdate (1 g in 100 mL of 50 mM H_2SO_4) were added, and the absorbance was determined at 700 nm. Since components of the reaction mixture (probably ATP and PEP) are not stable in acid molybdate and slowly liberate inorganic phosphate, several absorbance readings were taken at 5-min intervals and the values extrapolated to zero time. The absorbance was calibrated with standard curves run on known phosphate solutions. Assays run under these conditions can determine as low as 10 μM phosphate.

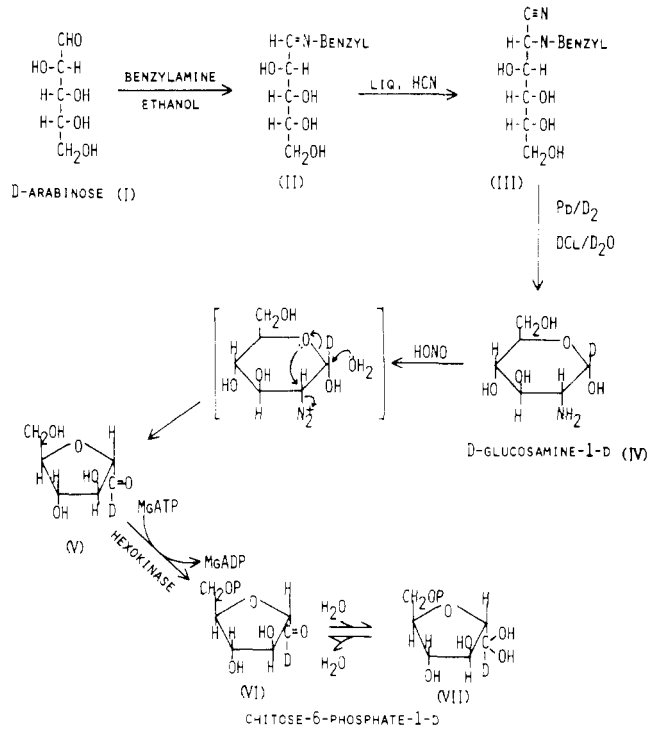


FIGURE 1: Synthetic scheme for preparation of chitose-*1-d* 6-phosphate.

Synthesis of Chitose-1-d 6-Phosphate. 2,5-Anhydro-D-mannose-*1-d* (chitose-*1-d*) was prepared according to the synthetic scheme in Figure 1. D-Glucosamine-*1-d* was prepared by a modification of the procedure of Kuhn & Kirschenlohr (1956). D-Arabinose (25 g) was added to 60 mL of ethanol and 20 mL of benzylamine and the mixture was heated on a steam bath until the crystals dissolved. Water-free liquid HCN (12 mL), prepared by the method of Ziegler (1932), was added to the cooled *N*-benzyl-D-arabinosamine (II) solution with stirring. The resulting *N*-benzyl nitrile (III) crystallized immediately. The solution was cooled, filtered, and washed with ethanol. Recrystallization from ethanol gave a white powder (mp 125–126 °C) which migrated as a single spot on TLC plates. About 8 g of the nitrile was dissolved in 140 mL of 0.5 N DCl in D_2O and 4 g of 5% palladium on barium sulfate was added. Catalytic deuteriogenation was run on a Parr apparatus at ~30 lb of D_2 gas for 24 h. When the deuteriogenation was run in H_2O, the product was only about 50% deuterated. The reaction mixture was filtered, concentrated, and crystallized from ethanol. The resulting product (compound IV) cochromatographed with an authentic sample of glucosamine on TLC plates.

2,5-Anhydro-D-mannose-*1-d* was prepared from glucosamine-*1-d* by nitrous acid treatment (Horton & Philips, 1973). Small quantities of chitose 6-phosphate were prepared by phosphorylation with ATP, catalyzed by yeast hexokinase under the following conditions: 100 mM Hepes (pH 8.0), 1 mM ATP, 3 mM $Mg(OAc)_2$, 1 mM phosphoenolpyruvate, 0.4 mM NADH, 0.25 mg of pyruvate kinase and lactate dehydrogenase, and 1–2 units of hexokinase. The production of chitose 6-phosphate was followed by the decrease in absorbance at 340 nm. It was found to be more practical when examining the kinetics of the induced ATPase reaction of fructose-6-P kinase to phosphorylate chitose in the cuvette prior

[2] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; PEP, phosphoenolpyruvate.

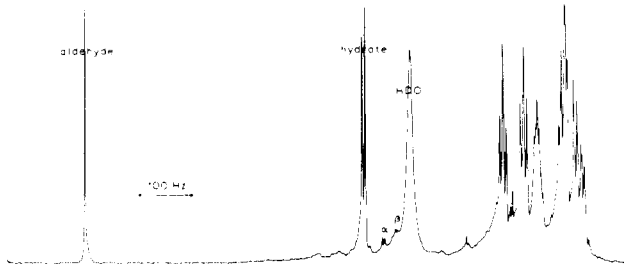


FIGURE 2: Proton NMR spectrum (270 MHz) of chitose. The region shown is from 3 to 10 ppm downfield from Me_4Si. The integrated areas of the peaks labeled aldehyde and hydrate were used to calculate the hydration equilibrium of chitose. Peaks labeled α and β are from the anomeric protons of contaminating glucose, which was removed by subsequent purification. Data acquisition conditions were the following: spectrum width = 1200 Hz, 45° pulse = 10 μs, recovery time = 10 s, number of scans = 200.

to the addition of the enzyme to start the reaction, since this provided a direct determination of the concentration present from the ADP formed.

Purity and Purification of Chitose and Chitose-1-d. Chitose-*1-d* was prepared as described above and stored at −20 °C as a concentrated syrup. No success has been reported with numerous attempts to crystallize chitose (Horton & Philips, 1973) so the concentration and purity of the product were determined by chemical and enzymatic means. Anhydrosugar alcohols such as chitose are cleaved slowly by periodate, while sugars with free adjacent hydroxyl groups are cleaved fairly rapidly. Treatment of chitose-*1-d* with periodate resulted in a rapid formation of iodate, indicating the presence of contaminating sugars. This was confirmed by NMR studies such as that in Figure 2 which showed peaks corresponding to the anomeric protons of glucose. Contamination with sugars which are substrates for yeast hexokinase will result in an inaccurate calibration of the concentration of chitose-*1-d*-6-P, and this inaccuracy will be reflected in the subsequent isotope-effect determinations.

Chitose and chitose-*1-d* were assayed for the presence of contaminating glucose and fructose after phosphorylation with hexokinase. Glucose-6-P dehydrogenase was added to the assay mixture and the levels of glucose-6-P present were determined from the amount of NADPH formed. After this reaction had gone to completion, phosphoglucoisomerase was added to convert any fructose-6-P present to glucose-6-P. The lack of additional absorbance changes indicated the absence of contaminating fructose, but the chitose-*1-d* preparation contained 15–20% contaminating glucose, while unlabeled chitose, prepared from glucosamine by the same procedure, contained less than 1% glucose.

Contaminating glucose was removed by the addition of 1 mL (1200 units) of glucose oxidase and 25 μL (7000 units) of catalase to 3 mL of stock chitose or chitose-*1-d* solution (100–500 mM). After the mixture was stirred at 0 °C for 3 h, the reaction was terminated by addition of several drops of CCl_4 and 10 mL of ethanol. The solution was vortexed, filtered to remove precipitated proteins, and concentrated to a thick syrup by rotary evaporation. This solution was run through small columns (prepared in Pasteur pipets) of Dowex-50 (H^+ form) and Dowex-3 (free base) to remove ionic contaminants. Solutions of chitose and chitose-*1-d* treated in this manner were free of glucose as determined by enzymatic assay. It is clear from the lack of any V isotope effect (see Results) that no other sugars were present which are substrates for the hexokinase reaction and whose 6-phosphates are inhibitors of fructose-6-P kinase. In particular, mannose was not present, since addition of mannose to chitose resulted in inhibition of the ATPase reaction by the mannose-6-P formed by the hexokinase reaction. Before the contaminating glucose was removed, however, an apparent V isotope effect was seen because of the much greater amount of glucose in the deuterated preparations as compared to the unlabeled chitose preparations.

Data Analysis. All least-squares fits of the data were performed by using Fortran programs which assume equal variance for the velocities (Cleland, 1979). Initial velocity patterns were fitted to eq 1, where v is the experimentally

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

determined velocity, V is the maximum velocity, A and B are the substrate concentrations, K_a and K_b are the respective Michaelis constants, and K_{ia} is the dissociation constant of A. Competitive inhibition data were fitted to eq 2, where K_{is} is

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (2)$$

the slope-inhibition constant and K is the apparent Michaelis constant. Noncompetitive inhibition data were fitted to eq 3,

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (3)$$

where K_{ii} is the intercept inhibition constant. V/K isotope-effect data were fitted to eq 4, where F_i is the fraction of

$$v = \frac{VA}{K(1 + F_i E_{V/K}) + A} \quad (4)$$

deuterium label, and $E_{V/K}$ is the isotope effect minus one.

Results

Induced ATPase Activity of Fructose-6-P Kinase. While attempting to use fructose-6-P kinase to determine the possible contamination of chitose-6-P with fructose-6-P or other phosphorylatable compounds, we observed that the reaction rates did not show the typical asymptotic approach to completion at low levels of chitose 6-phosphate but remained linear. The slow rates observed were not stoichiometric with the amount of chitose 6-phosphate present but continued virtually unchanged even after the amount of ADP produced was three to four times the initial concentration of chitose 6-phosphate. The rate of production of ADP in the presence of chitose 6-phosphate was found to increase linearly with the amount of fructose-6-phosphate kinase added and was dependent on the levels of chitose 6-phosphate and MgATP. This implies the presence of an ATPase activity in fructose-6-P kinase induced by the binding of chitose-6-P.

If chitose-6-P is phosphorylated to an unstable diphosphate, there should be a lag in the rate of production of inorganic phosphate, with the lag proportional to the steady-state level of chitose diphosphate. When the reaction was followed by using a coupled assay for the formation of inorganic phosphate, no lags were observed, indicating that if the diphosphate product is being formed and decomposes nonenzymatically, the steady-state level present is not detectable by this technique. When the experiment was repeated under conditions where the nonenzymatic breakdown of the diphosphate should be slower relative to its enzymatic rate of formation (0 °C and 100 units of fructose-6-phosphate kinase), no lag was seen in the production of phosphate and no burst was seen in the initial utilization of ATP as measured by the formation of ADP.

An initial velocity pattern, varying the levels of chitose-6-P at several fixed levels of MgATP, is shown in Figure 3. These

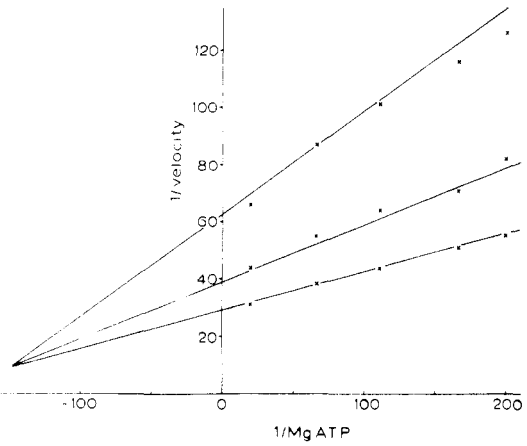


FIGURE 3: Initial velocity pattern for ATPase reaction of fructose-6-phosphate kinase at pH 8. MgATP was varied from 5 to 50 μM at 0.11, 0.23, and 0.42 mM chitose 6-phosphate. The reaction was followed by coupling the production of MgADP with pyruvate kinase and lactate dehydrogenase. The lines drawn through the data points are from a fit to eq 1.

Table I: Kinetic Parameters for Fructose-6-phosphate Kinase

parameter	fructose 6-phosphate[a]	2,5-anhydro-mannitol-6-P[a]	chitose-6-P[b]
V_{max} (μmol/min)	0.766	1.01	0.023
K_{MgATP} (μM)	27 ± 3	20 ± 8	3.1 ± 1.2
$K_{i,MgATP}$ (μM)	(1 ± 3)	(8 ± 7)	6.7 ± 1.5
$K_{sugar\text{-}P}$ (μM)	70 ± 9	150 ± 52	300 ± 50
$K_{i,\ sugar\text{-}P}$ (μM)	(2 ± 8)	(58 ± 55)	650 ± 320
Fructose-1,6-P_2 Inhibition vs. Sugar-P[c]			
K_{is} (mM)	0.71 ± 0.15		4.1 ± 0.20
Fructose-1,6-P_2 Inhibition vs. MgATP[d]			
K_{is} (mM)	1.1 ± 0.4		0.89 ± 0.07
K_{ii} (mM)	1.3 ± 0.3		4.1 ± 0.40

[a] Kinetic parameters were determined at pH 7.4 by Bar-Tana & Cleland (1974). [b] Kinetic parameters were determined at pH 7.9. [c] MgATP was present at 20 μM with fructose 6-phosphate as the substrate and was present at saturating levels (1 mM) with chitose 6-phosphate as the substrate. [d] Fructose 6-phosphate and chitose 6-phosphate were each present at 100 μM.

data were fitted to eq 1, and the resulting kinetic parameters are compared in Table I to the values reported by Bar-Tana & Cleland (1974) with fructose-6-P and 2,5-anhydromannitol-6-P as substrates. Fructose-1,6-P was used as an inhibitor against chitose-6-P or MgATP to determine if chitose-6-P was binding at the active site of fructose-6-P kinase. The data were fitted to eq 2 (competitive inhibition) and eq 3 (noncompetitive inhibition), respectively. The fitted inhibition constants are compared (in Table I) to the values with fructose-6-P as substrate.

The hydration equilibrium of chitose was determined by proton NMR spectroscopy. The 270-MHz proton NMR spectrum of chitose is shown in Figure 2. The recovery time between pulses was long to allow complete return of the peaks to their equilibrium position and therefore allow accurate determination of the population of species. The ratio of the areas of the aldehyde and hydrate peaks indicated that chitose is 60% hydrated in D_2O. This value must be corrected for a 15% deuterium solvent isotope effect on the hydration equilibrium (Gruen & McTigue, 1963) to give a value of 52% hydrate and 48% free aldehyde for chitose in water (hydrate/aldehyde = 1.083). Deuteration at C-1 causes a 37% isotope effect on the hydration equilibrium, giving a hydrate/aldehyde ratio of 1.083 × 1.37 ≈ 1.484 or 59.7% hydrate and 40.3% free aldehyde for chitose-*1-d*.

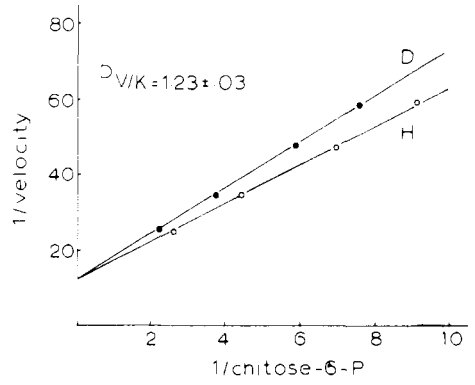


FIGURE 4: Observed deuterium isotope effect with chitose-*1-d* 6-phosphate for the ATPase reaction catalyzed by fructose-6-phosphate kinase at pH 8. The concentrations of chitose and chitose-*1-d* were determined by end-point assays with hexokinase. The lines drawn through the data are from a fit to eq 4.

Table II: Determination of Inorganic Phosphate Produced during the Phosphorylation of Chitose

enzyme	concentration of P_i (μM)[a] + enzyme	− enzyme	difference	excess ADP[b] (μM)
fructokinase I	60	20	40	36
II	58	17	41	38
hexokinase	28	22	6	-

[a] The concentration of inorganic phosphate was determined by the phosphomolybdate assay and quantitated by comparison to standard curves. [b] Determined by following the production of ADP in the reaction mixtures by a coupled pyruvate kinase–lactate dehydrogenase assay prior to determination of inorganic phosphate. The values reported are the difference between the ADP produced by the fructokinase-catalyzed reactions and that produced by the hexokinase-catalyzed reactions.

In order to identify the active species of chitose-6-P, the rate of production of ADP was examined with either chitose-6-P or chitose-6-P-*1-d* (Figure 4). A normal isotope effect on V/K of 1.23 ± 0.03 was observed, compared to an expected value of 0.48/0.403 = 1.19 if the free aldehyde were the active species, but no effect was seen on V.

Induced ATPase Activity with Fructokinase. For verification of the absence of contaminating sugars which might be phosphorylated by the less specific hexokinase, the concentrations of chitose and chitose-*1-d* were also determined with fructokinase, which is specific for β-furanose sugars and analogues containing a similar ring structure (Raushel & Cleland, 1977). Instead of giving an absorbance change corresponding to a concentration which was the same or lower than that which was observed with hexokinase, fructokinase gave a 25% greater absorbance change, and, as shown in Table II, inorganic phosphate was also formed in a level equal to the ADP in excess of that from the hexokinase reaction. Chitose thus appears to induce ATPase activity as well as being phosphorylated.

Discussion

ATPase Activity of Fructose-6-P Kinase. The maximum velocity of the induced ATPase activity is slow compared to the kinase reaction with fructose-6-P or 2,5-anhydromannitol-6-P as substrates. Catalysis should be rate limiting, and the K_i values determined from initial velocity studies should equal the thermodynamic binding constants. Kemp & Krebs (1967) determined a binding constant of 4–5 μM for

MgATP binding to the free enzyme by the gel filtration method. The value of 6.7 ± 1.5 μM from our kinetic studies of the ATPase reaction is in good agreement, and it is probable that such experiments provide the easiest and most precise way to determine the dissociation constant for MgATP.

The possibility that chitose 6-phosphate is binding at an effector site to induce the ATPase activity is diminished by the competitive inhibition seen with fructose 1,6-bisphosphate. The binding of chitose 6-phosphate is thus probably at the active site. MgADP was a noncompetitive inhibitor against chitose 6-phosphate, consistent with the random mechanism postulated for fructose-6-phosphate kinase (Bar-Tana & Cleland, 1974), with a dead-end E–MgADP–chitose 6-phosphate complex forming.

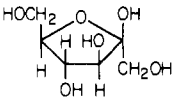
The normal V/K isotope effect observed with chitose-*1-d*-6-P is the value expected if only the free aldehyde were the activator. Further, the absence of any V isotope effect shows that the hydrate does not bind appreciably, since a normal V isotope effect would result from the increased ratio of hydrate to free aldehyde in the deuterated chitose 6-phosphate if both were bound with near-equal affinity. This observation places narrow constraints on the geometry of the sugar phosphate binding site. Since the hydrated form of chitose 6-phosphate does not bind to the enzyme and the aldehyde plus a water molecule would occupy more space than the hydrate alone, it would not be realistic to postulate a mechanism in which the bound aldehyde induces a phosphorylation of water by ATP in the active site. The most likely explanation for the ATPase reaction is that chitose 6-phosphate acts as a phosphoryl acceptor.[3] The phosphorylation of the oxygen of a carbonyl group has been demonstrated by Satterthwait & Westheimer (1980), who observed that methyl metaphosphate, generated in solution, will react with acetophenone in the presence of base to yield an enol phosphate.

In the catalytic conformation of the enzyme the phosphorylated aldehyde is presumably not accessible to solvent, but after it is released from the enzyme solvent attack at the carbonyl carbon would allow release of phosphate and the regeneration of chitose 6-phosphate. Satterthwait & Westheimer (1980) have shown in model studies that if the phosphorylation of acetophenone by methyl metaphosphate is run in the presence of aniline, a Schiff's base is formed which probably results from nucleophilic attack on the carbonyl carbon of a phosphorylated intermediate and subsequent release of phosphate. Alternately, solvent attack on the phosphorus atom of the phosphorylated aldehyde while it was still bound to the enzyme and the carbonyl carbon inaccessible to solvent but the nucleotide has been released could also lead to the observed products. These possibilities could be distinguished by looking for ^{18}O transfer from aldehyde to phosphate during the ATPase reaction.

It is also possible that the aldehyde is not phosphorylated but merely induces the enzyme to catalyze the decomposition of ATP to ADP and metaphosphate. If the latter were released as such into solution, it would react immediately with solvent. Such a mechanism could be tested for by looking for reaction of metaphosphate with methanol or a similar alcohol present in relatively high concentrations in solution. Methyl phosphate formed in such a way from chiral [γ-^{16}O,^{17}O,^{18}O]ATP would probably be racemic.

Further experiments are thus clearly required to determine the exact chemical mechanism of this very interesting induced ATPase reaction. However, the basic principle of using isotope effects with deuterated aldehydes to determine the nature of the activation is clearly practical and can be applied to other aldehyde-induced ATPase activities such as those of fructokinase and glycerokinase.

ATPase Activity of Fructokinase. The activity of chitose as both an inducer of ATPase and as a substrate for the kinase activity of fructokinase can be explained if chitose binds to fructokinase in two ways. When binding as an analogue of fructose (VIII) or 2,5-anhydromannitol (IX), which is an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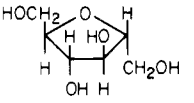
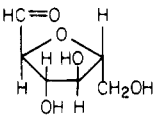
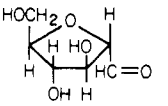

IX


X


XI

excellent substrate for fructokinase, chitose (X) is phosphorylated in the same manner as the normal substrates. However, if chitose binds with the aldehyde group in the position where the 1-hydroxymethyl group normally binds (XI), there will be an ATPase activity induced similar to that seen with fructose-6-P kinase.

If the binding of chitose to fructokinase is mutually exclusive in the two binding modes, then the ratio of kinase to ATPase activities will be constant at all substrate concentrations. This can be shown for a simple model:

$$P + E \xleftarrow{k_3} EA \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} E \underset{k_6}{\overset{k_5 A}{\rightleftharpoons}} E\bar{A} \xrightarrow{k_7} E + Q \qquad (5)$$

where A is chitose, P is chitose-6-P, Q is inorganic phosphate, EA is an enzyme–chitose binary complex with chitose binding as an analogue of fructose (X), and E$\bar{A}$ is the enzyme–chitose complex with chitose binding to induce an ATPase activity (XI).[4] The rates of formation of P and Q in this system are given by

$$\frac{dP}{dt} = \frac{(V/K_p)(A_0 - P)}{1 + (1/K_p + 1/K_q)(A_0 - P)} \qquad (6)$$

$$\frac{dQ}{dt} = \frac{(V/K_q)(A_0 - P)}{1 + (1/K_p + 1/K_q)(A_0 - P)} \qquad (7)$$

where

$$K_p = (k_2 + k_3)/k_1 \qquad K_q = (k_6 + k_7)/k_5 \qquad (8)$$

$$(V/K_p) = k_1k_3/(k_2 + k_3) \qquad (V/K_q) = k_5k_7/(k_6 + k_7) \qquad (9)$$

[3] A reviewer has raised the possibility that the aldehyde forms an aldimine with a lysine in the active site and the water molecule produced attacks ATP. While we cannot rule this out at present, we think it unlikely, since any lysines present would be protonated and hydrogen bonded to the γ-phosphate oxygens of ATP and their pKs would be high enough that aldimine formation would be unfavorable. Also it is questionable whether such a mechanism is sterically possible, since lysines would be next to the γ-phosphate oxygens of ATP and not the CH_2 group of the alcohol substrate or the carbonyl carbon of the aldehyde which should occupy the same location in the active site.

[4] If mechanism 5 is made more complex by adding additional steps after k_3 and k_7 in each path or by expanding k_3 and k_7 to include several reversible steps prior to product release, eq 6 and 7 are still obtained, although the definitions of the kinetic constants in eq 8 and 9 become more complex. The situation is similar to that involved in determining tritium isotope effects by competition between tritiated and unlabeled substrate molecules. In that case, also, only relative V/K effects are seen regardless of the complexity of the mechanism.

and A_0 is the initial concentration of A.[5] Since eq 6 and 7 have the same denominator

$$\frac{dQ}{dP} = \frac{V/K_q}{V/K_p} \quad (10)$$

and the ratio of products formed is constant regardless of the concentration of chitose present at any time in the course of the reaction. If eq 10 is integrated from zero time (when P and Q are zero) to infinite time (at which point P and Q have their final values after all chitose has been phosphorylated), one obtains

$$\frac{Q_\infty}{P_\infty} = \frac{V/K_q}{V/K_p} \quad (11)$$

and thus the ratio of total products finally formed is also the ratio of V/K values. Since about 25% more ADP was formed with fructokinase than with hexokinase, V/K for the ATPase reaction is 25% that for the kinase reaction. This simultaneous ATPase and kinase activity may also occur to a small extent when chitose is a substrate for hexokinase (see Table II), since some inorganic phosphate was formed during phosphorylation.

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[5] Note that when either dP/dt or dQ/dt is measured, the apparent Michaelis constant will be the same, namely, $1/(1/K_p + 1/K_q)$. The V_{max} for kinase activity will be $k_3/(1 + K_p/K_q)$, while that for the ATPase activity will be $k_7/(1 + K_q/K_p)$.

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Nuclear Magnetic Resonance Studies of Carbonic Anhydrase Catalyzed Reversible Hydration of Acetaldehyde by the Saturation Transfer Method†

Dalia Cheshnovsky and Gil Navon*

ABSTRACT: The catalysis of the chemical exchange between acetaldehyde and its hydrate by bovine carbonic anhydrase B was investigated by the NMR line broadening and saturation transfer techniques. While both chemical exchange and binding to the enzyme had an effect on the line broadening of acetaldehyde and its hydrate, the saturation transfer method enabled us to measure the exchange without the interference from the effect of the binding. *p*-Toluenesulfonamide and azide ion were found to inhibit completely the carbonic anhydrase catalyzed exchange reaction. However, there was a residual line broadening of the acetaldehyde by the enzyme in their presence. This was interpreted as binding to sites other than the active site of the enzyme. The pH profile of the catalysis of the reversible hydration of acetaldehyde by carbonic anhydrase in the absence of a buffer indicates high- and low-activity forms of the enzyme with a transition at pD 7.65. The cadmium(II) enzyme was found to be inactive, and the cobalt(II) derivative was found to have similar activity to that of the native system.

The enzyme carbonic anhydrase catalyzes the interconversion of carbon dioxide and bicarbonate at a rate which is one of the fastest known for enzyme catalysis [for recent reviews, see Wyeth & Prince (1977) and Pocker & Sarkanen (1978)]. The enzyme is capable of catalyzing other reactions such as hydrolysis of some carboxylic esters (Armstrong et al., 1966) and sulfonic and carbonic esters (Pocker & Stone, 1968a; Pocker & Guilbert, 1974) and the reversible hydration of aldehydes and pyruvic acid (Pocker & Meany, 1965, 1967a,b, 1970), although with much smaller rates as compared with that of the hydration of CO_2.

Since the process of obtaining NMR spectra is relatively slow, NMR investigations of the binding of small molecules to enzymes are limited mostly to inhibitors, activators, or very slowly reacting substrates (Dwek, 1973; Mildvan, 1974; James,

† From the Department of Chemistry, Tel-Aviv University, Tel Aviv, Israel. *Received October 9, 1978.* This research was supported by a grant from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel.

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