Use of Chromium–Adenosine Triphosphate and Lyxose to Elucidate the Kinetic Mechanism and Coordination State of the Nucleotide Substrate for Yeast Hexokinase†

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ABSTRACT: When initial velocities are measured with yeast hexokinase at pH 7, 17°, the inert coordination complex chromium–ATP is competitive *vs.* MgATP and noncompetitive with glucose, with a dissociation constant of 4–6 μM in either the presence or absence of glucose. These patterns confirm a random kinetic mechanism for this enzyme. With CrATP present, however, the reaction slows down over the first several minutes to a much slower rate, suggesting tighter binding of CrATP with time. When CrATP, MgATP, and D-lyxose are preincubated with the enzyme for 10 min and the reaction started by addition of excess glucose, the dissociation constant of CrATP is now 0.13 μM and the reaction is linear with time. When glucose, CrATP and enzyme are incubated together and then placed on a Sephadex column, 1 mol each of CrATP and glucose per active center is tightly bound to the enzyme, thus providing a simple and precise method of determining the concentration of active sites. This tight complex, after denaturation with acid, releases 25% free glucose and 75% of a chromium complex containing both ADP and sugar-6-P. CrADP–glucose-6-P is also slowly released from the enzyme during incubation, so that CrATP is actually a very slow substrate. Binding of CrATP with the formation of CrADP–sugar-6-P complexes is also induced by mannose, fructose, glucosamine, 2,5-anhydro-D-glucitol, 2,5-anhydro-D-mannose, and 2,5-anhydro-D-mannitol, while glucose-6-P, 6-deoxyglucose, and lyxose also induce tight binding of CrATP. With excess enzyme, only 25% of CrATP is bound, and the rest does not inhibit the hexokinase reaction. Since bidentate $Cr(NH_3)_4ATP$ and monodentate CrADP also display inhibition which is tighter with time, but since bidentate CrADP is a poor inhibitor, the actual substrates in the hexokinase reaction appear to be β,γ-bidentate MgATP and β-monodentate MgADP. Tighter inhibition by Cr-8-BrATP than by CrATP suggests that ATP assumes the syn conformation on the enzyme. The substrate inhibition by MgATP induced by the presence of lyxose is shown to be competitive *vs.* glucose and partial, and, together with other data available, to suggest a kinetic mechanism that is random, but where (1) the rate constant for release of glucose from E–glucose is equal to V_{max}, and that for release of glucose from central complexes is less than V_{max}; (2) the majority of the reaction flux when both substrates are present at K_m levels goes through the path with glucose adding before MgATP, but where at physiological levels the flux through the two paths is more equal; (3) a conformation change occurs when both sugar and nucleotide are present which tightens binding by 40-fold, with the change being rapid with Mg nucleotides, and slow with Cr nucleotides. The preparation of bidentate $Cr(NH_3)_4ATP$, $Cr(NH_3)_4$–8-BrATP, $Cr(NH_3)_4$–adenylyl imidodiphosphate, and $Cr(NH_3)_4PP$ and monodentate $Cr(NH_3)_4(H_2O)ATP$, $Cr(NH_3)_5ATP$, and $Cr(NH_3)_5PP$ (the latter nicely crystalline) is reported, as well as the preparation of CrAMP, CrADP–P_i, and of chromium complexes of 8-BrATP, adenosine tetraphosphate, adenylyl imidodiphosphate, and α,β- and β,γ-methylene ATP.

Yeast hexokinase, which catalyzes the phosphorylation of glucose to glucose 6-phosphate (glucose-6-P), is one of the most studied kinases, but its kinetic mechanism is still the subject of dispute. Fromm and Zewe (1962) on the basis of inhibition data suggested that the mechanism was random, and Fromm *et al.* (1964) showed that the mechanism was not a rapid equilibrium one, but that the release of sugar or sugar phosphate was at least partly rate limiting. The evidence for the random mechanism has been summarized by Purich *et al.* (1973). On the other hand, Hammes and Kochavi (1962) postulated that the mechanism was ordered, with glucose combining first, but their product inhibition data were equally consistent with the random mechanism. More recently Noat *et al.* (1968) have again proposed the ordered mechanism on the basis that the apparent K_i for MgATP varied with the sugar being phosphorylated, a result inconsistent with a rapid equilibrium random mechanism. However, Bar-Tana and Cleland (1974) have recently pointed out that in random mechanisms where the rate constants for dissociation of the substrates from the enzyme are less than V_{max} (as is the case here for glucose) the initial velocity patterns are distorted, so that incorrect values for the apparent dissociation constants of the substrates from their binary complexes with enzyme are observed (this is true of MgATP with hexokinase). As a result of these questions it appears that independent kinetic evidence, in addition to the isotopic exchange (Fromm *et al.*, 1964) and inhibition experiments that support the random mechanism (Fromm and Zewe, 1962; Fromm, 1969; Kosow and Rose, 1970; Rudolph and Fromm, 1971b), would be helpful in settling the matter.

In this laboratory we have recently prepared inert coordination complexes of Cr(III) and various nucleotides for use as dead end inhibitors in kinetic studies (DePamphilis and Cleland, 1973). If the mechanism of yeast hexokinase were

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ordered with glucose adding first, CrATP should be a competitive inhibitor of MgATP, but an uncompetitive inhibitor of glucose, while if the mechanism were random, CrATP would be noncompetitive *vs.* glucose.[1] This paper presents evidence that CrATP is indeed competitive *vs.* MgATP and noncompetitive *vs.* glucose, as expected for the random mechanism.

During these studies we also observed that the degree of inhibition by CrATP increased with time, with the initial rate decreasing over several minutes to a slower rate that then did not change until considerable reaction had taken place. This process appears to result from a conformation change induced by the presence of the sugar, and in the resulting E-glucose-CrATP complex, 1 mol each of glucose and CrATP per active center is tightly bound to the enzyme, and some phosphorylation to give CrADP-glucose-6-P occurs. The specificity of chromium nucleotides which permits this conformation change suggests that the substrates for hexokinase are β,γ-bidentate MgATP and β-monodentate MgADP, and that the nucleotide exists in the syn conformation.

It was first noted by Rudolph and Fromm (1971a) that D-lyxose induced substrate inhibition by MgATP. As part of the present work we have determined the form of this substrate inhibition to be competitive, but partial, as is predicted for a random mechanism in which lyxose binds appreciably only to E-MgATP, but most of the reaction flux at K_m levels of the substrates goes by the path where glucose adds before MgATP. A kinetic mechanism consistent with all data available is presented.

Materials and Methods

Yeast hexokinase and glucose-6-P dehydrogenase were obtained from Boehringer or Sigma. D-Lyxose (Sigma) contained 0.026% glucose which for experiments with over 50 mM lyxose was removed by incubation of a 500 mM solution with glucose oxidase until glucose was absent, followed by evaporation and crystallization from ethanol. 2,5-Anhydro-D-mannose was synthesized by nitrous acid treatment of glucosamine, and 2,5-anhydro-D-mannitol by subsequent reduction with borohydride at pH 7 (Bera *et al.*, 1956). 1,5-Anhydro-D-mannitol was prepared by treatment of D-mannitol with HCl (Fletcher, 1963), and 2,5-anhydro-D-glucitol by anhydridization of 1,6-dibenzoylmannitol (Hockett *et al.*, 1946). 1,5-Anhydro-D-glucitol prepared by the method of Ness *et al.* (1950) contained 0.5% glucose, which was removed by treatment with glucose oxidase. 1-Deoxy-D-fructose was prepared by the method of Ishizu *et al.* (1967), and 2,5-anhydro-D-lyxitol according to Defaye (1964).

Chromium Complexes. CrATP, $Cr(NH_3)_2ATP$, and $Cr(NH_3)_3ATP$ were prepared by the aniline procedure of DePamphilis and Cleland (1973), as were the chromium complexes of 8-BrATP (Schwarz/Mann) and of the ATP analogs, adenylyl imidodiphosphate and α,β- and β,γ-methylene ATP (α,β-methylene ATP from Sigma, the other two from P-L Biochemicals). These latter complexes had spectra and properties similar to those of CrATP. Bidentate CrADP, $Cr(NH_3)_3ADP$, and $Cr(NH_3)_4ADP$ were prepared by the methods of DePamphilis and Cleland (1973). Bidentate $Cr(NH_3)_4ATP$ was prepared by heating a solution 10 mM each in $[Cr(NH_3)_4Cl(H_2O)]Cl_2$ (Pfeiffer, 1905) and Na_2ATP at 80° for 8 min. After cooling, the red solution was adsorbed on Dowex 50-X2-H^+, 100–200 mesh, and $Cr(NH_3)_4ATP$ (33% yield) was eluted as a focused band with 0.1 M lithium formate (pH 3.5). Monodentate $Cr(NH_3)_4(H_2O)ATP$ (29% yield) was then eluted as a diffuse band with 0.1 M lithium formate (pH 4.5). Each band was adjusted to pH 2, adsorbed again on Dowex 50, and eluted with 0.1 M aniline. After extraction with ether (3 × 10 volumes), the isoionic pH values were 3.3 and 4 for the bidentate and monodentate compounds. These values prove the γ-phosphate is coordinated; if it were free, the isoionic pH values would be around 5 (coordination of the β- and γ-phosphates changes the secondary phosphate pK from above 6 to below 3, while if only the γ-phosphate is coordinated, the negative charge on the β-phosphate raises the pK to near 4; the adenine pK is around 3.7 in all of these compounds). The visible spectra of these compounds are identical (λ_{max} 385 (ϵ 32), 520 (ϵ 44)), and they both contain three phosphates per adenine. The monodentate complex changes slowly into the bidentate one in the icebox (50% in 1 week at pH 4), but the bidentate complex appears stable. $Cr(NH_3)_4$-8-BrATP and $Cr(NH_3)_4$-adenylyl imidodiphosphate were prepared by a similar procedure. When $[Cr(NH_3)_5H_2O](NO_3)_3 \cdot NH_4NO_3$ (Mori, 1957) was used in a similar preparation, 21% of $Cr(NH_3)_4ATP$ was eluted at pH 3.5 and 40% of $Cr(NH_3)_5ATP$ at pH 4.5. This latter compound was orange (λ_{max} 372 (ϵ 31), 506 (ϵ 47)) and stable with an isoionic pH of 4.0. The $Cr(NH_3)_4ATP$ from the two preparations appeared identical, but as can be seen from Table III, did not equally inhibit hexokinase, and may contain different proportions of the two possible coordination isomers. When disodium pyrophosphate replaced ATP in the pentaammine preparation, $Cr(NH_3)_4PP$ (isoionic pH, 3.15) and $Cr(NH_3)_5PP$ (isoionic pH 4.4) were obtained. The first was focused from Dowex 50 with pH 3.5 formate, and the latter was eluted with 0.1 M sodium formate. Both compounds were readsorbed on Dowex 50 after reacidification and eluted with aniline for final purification. Crystals of isoionic $Cr(NH_3)_5PP$ formed in a 25 mM solution at 4°, and crystallization was accomplished by addition of 1 vol of ethanol.

Chromium-adenosine tetraphosphate was prepared by the method used for CrATP, and was passed through Dowex 50-H^+ without being adsorbed (a small amount of CrATP was adsorbed). It was adsorbed to Dowex 1-X2-Cl as a tight green band and eluted with 0.1 M HCl. Its spectrum is similar to that of CrATP.

Monodentate CrADP was prepared by heating a solution 20 mM each in $CrCl_3$ and NaADP for 1 min at 80°, followed by quick cooling. The solution was then adsorbed on Dowex 50-X2-H^+ and washed with 0.1 N HCl until the adenine content of the eluate dropped below 200 μM (this removes CrATP and most of the unreacted ADP). After a water wash, the column was eluted with 0.1 M sodium acetate (pH 4.3) and monodentate CrADP (10% yield; characterized by its inhibition *vs.* hexokinase) emerged with the pH front. When the heating period was 10 min, the CrADP behaved similarly on the column, but did not inhibit hexokinase. Since the spectra of mono- and bidentate CrADP ap-

[1] An uncompetitive inhibition is one which is seen at high levels of variable substrate, but not at near zero levels; a noncompetitive inhibition is seen at both high and low levels of variable substrate, although the strength of the inhibitions may be different. Uncompetitive inhibition is seen in the ordered case since CrATP (and thus presumably MgATP) cannot combine with free enzyme, but only with E-glucose, which disappears as the glucose concentration is extrapolated to zero. In the random case CrATP can combine with free enzyme as well as with E-glucose, and thus inhibits at both high and low glucose.

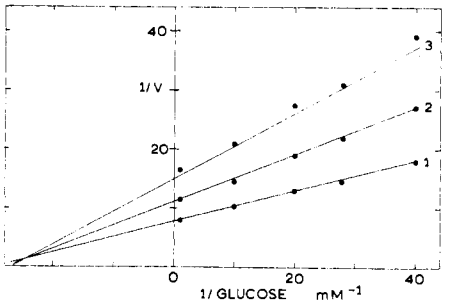


FIGURE 1: Noncompetitive inhibition of initial velocities by CrATP *vs.* glucose: MgATP, 40 μM, CrATP: (1) 0; (2) 2.5 μM; (3) 5 μM.

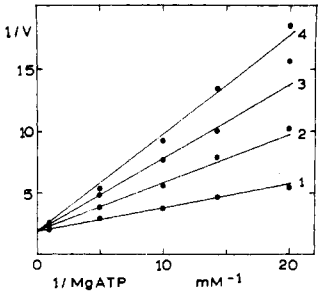


FIGURE 2: Competitive inhibition of initial velocities by CrATP *vs.* MgATP: glucose, 10 mM; CrATP: (1) 0; (2) 5 μM; (3) 10 μM; (4) 15 μM.

pear nearly the same, and their chromatographic behavior is so similar, their separation has not yet been achieved, but in the preparation of CrATP, 1-min heating gives twice as much mono- as bi- or tridentate material.

CrAMP was prepared by heating a solution 10 mM each in $CrCl_3$ and AMP at 80° for 8 min. It was adsorbed on Dowex 50-H^+, and eluted by 1.2 N HCl after $Cr(H_2O)_6^{3+}$ was removed with 0.6 N HCl. After readsorption on a small column of Dowex 50, it was eluted with 1.2 N $HClO_4$, neutralized to pH 3.3 with solid $KHCO_3$, cooled, and filtered. To prepare CrADP-P_i, freshly prepared CrADP was eluted from Dowex 50 with 0.4 M $HClO_4$, neutralized to pH 3 with solid K_2HPO_4, cooled, filtered, heated at 80° for 5 min, and adsorbed on Dowex 50-H^+ as a focused band below the clear area containing K^+. When the column was washed with water, apple green CrADP-P_i was eluted as the pH reached 2.2 (the pH changes from 1.2 to 3.1 as the phosphate concentration in the eluate drops). CrADP remained on the column. The visible spectrum of CrADP-P_i has λ_{max} values of 624 and 440, and ϵ of both peaks about 30.

Kinetic Assays. Kinetic studies were run in 3.0 ml total volume in 1-cm cuvets by measuring absorbance changes at 340 nm with a Gilford optical density (OD) converter and a 10-mV recorder. Since the time course of the reaction is not linear in the presence of CrATP (see Figure 4), most kinetic studies were run at 17° in order to slow down the conformation change and permit initial velocities to be determined more accurately. Some studies were done at 25°, and in these cases the temperature is noted. The assay mixture for kinetic studies included 50 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0, 100 μM TPN, 6 units of glucose-6-P dehydrogenase, 1.4 units of hexokinase, 1–5 mM free Mg^{2+} (as acetate), and MgATP and glucose as specified. When ATP levels were 10 mM or higher, 50 units of glucose-6-P dehydrogenase were used.

Data Analysis. Kinetic data were plotted graphically to determine the pattern, and then fitted to the appropriate rate equation by the least-squares method, assuming equal variance for the velocities (Wilkinson, 1961), and using the Fortran programs of Cleland (1967). Data for linear competitive inhibition were fitted to eq 1, and for linear noncompetitive inhibition to eq 2. The points on the graphs are

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

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

the experimental data, and the lines are from the fits to eq 1 or 2.

Results

Inhibition of CrATP vs. Glucose and MgATP. When CrATP was used as an inhibitor *vs.* glucose and the reaction started by addition of hexokinase, the resulting pattern of initial velocities was noncompetitive (Figure 1). This pattern was repeated ten times at MgATP concentrations from 40 to 1000 μM, and the corrected values of the dissociation constants of CrATP in the absence and presence of glucose were obtained by dividing K_{is} or K_{ii} values from fits to eq 2 by $(1 + [MgATP]/K_{MgATP})$, where K_{MgATP} was taken to be 100 μM based on the average value observed in a number of initial velocity and inhibition experiments (K_m and apparent K_i are the same for MgATP and glucose, since as has been noted by all previous workers, the initial velocity pattern crosses on the horizontal axis). Weighted averages for the dissociation constants of CrATP in the absence and presence of glucose were 4.2 ± 0.3 and 3.9 ± 0.1 μM when the reciprocals of the squares of the standard errors were used as weights, but when the ratio of the standard error to the value was squared and inverted to give the weights, the resulting values were 6.1 ± 0.4 and 6.3 ± 0.2 μM. The dissociation constant of CrATP thus appears to be 4–6 μM, and to be unaffected by the presence of glucose. Since the CrATP used in these studies contains 20–25% of the active bidentate isomers (see below), the true dissociation constant of the active inhibitor is about 1 μM.

When MgATP was varied and the reaction started by addition of hexokinase, CrATP was a competitive inhibitor with K_{is} values of 2.8 ± 0.2, 4.9 ± 0.3, and 6.1 ± 0.9 μM in three experiments (Figure 2). These values agree with those derived above from the noncompetitive patterns *vs.* glucose. When MgATP, CrATP, and hexokinase were preincubated for 10 min with 16.7 mM lyxose, and the reaction then started by addition of 10 mM glucose, CrATP still appeared to be a competitive inhibitor *vs.* MgATP (although the long extrapolation involved does not make it possible to eliminate some effect on the intercepts), but the inhibition constant was now 0.13 ± 0.05 and 0.13 ± 0.01 μM in two experiments (Figure 3). The time course of the reaction was now linear and did not show the rapid slowing down to a lower velocity that was always seen when the reaction was run without preincubation.

Effect of Sugars on the Strength of CrATP Inhibition. As noted above, the time course of the reaction in the presence of CrATP showed a rapid decrease in initial velocity to a slower, but now more nearly linear rate (Figure 4). This phenomenon and the drastic drop in the K_i for CrATP induced by preincubation with lyxose indicate that glucose and lyxose cause an increase in the strength of CrATP binding that takes several minutes to become complete at

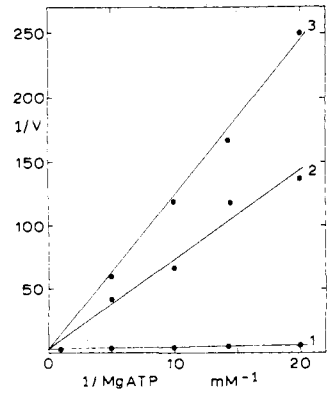


FIGURE 3: Competitive inhibition by CrATP *vs.* MgATP after preincubation in the presence of lyxose. MgATP, CrATP, 16.7 mM lyxose, and hexokinase were incubated 10 min, and the reaction then started by the addition of 10 mM glucose; CrATP: (1) 0; (2) 5 μM; (3) 10 μM.

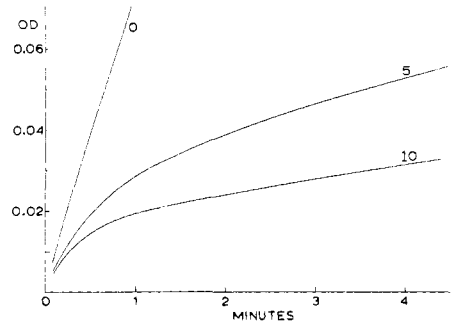


FIGURE 4: Time course of reaction in the presence of different levels of CrATP (μM): MgATP, 200 μM; excess Mg^{2+}, 5 mM; glucose, 1 mM.

17°. That this phenomenon can also be produced by other molecules that are either substrates or inhibitors for hexokinase was shown by incubating hexokinase with 20 μM CrATP, 200 μM MgATP, and the compound being tested (at about twice its dissociation constant in most cases) for various lengths of time at 25°, and then determining the initial velocities when 10 mM glucose was added (the assay determined only glucose-6-P, and not the products of phosphorylation of those sugars that were themselves substrates). In most cases the initial velocity decreased with time of preincubation and then leveled off (Table I), with those compounds that are the best substrates for hexokinase causing the most rapid change, and the greatest increase in degree of inhibition.

Where the sugar tested was a good substrate for hexokinase, however, it was only possible to see the effect if the increase in strength of CrATP binding took place rapidly compared with the rate of phosphorylation of the sugar. Thus, while 2,5-anhydro-D-glucitol, which has a V_{max} equal to that of glucose, but a very high K_m (47 mM (Raushel and Cleland, 1973), induces a rapid increase in the strength of the CrATP inhibition, and 2,5-anhydro-D-mannose (V_{max} equal to that of glucose, K_m = 0.3 mM (Raushel and Cleland, 1973)) induces a rapid, but smaller change, 2,5-anhydro-D-mannitol, which is a good substrate (K_m, 6 mM) with a V_{max} 50% more than that of glucose (Raushel and Cleland, 1973), does not induce a fast enough change to be observed, although the binding studies reported below show that it does induce tighter binding of CrATP. Neither 1,5-anhydro-D-mannitol nor 1,5-anhydro-D-glucitol, both of which are poor substrates for hexokinase (anhydromannitol: K_m, 7 mM; V_{max}, 6% that of glucose; anhydroglucitol: K_m, 3 mM; V_{max}, 1% that of glucose), induces any change in the strength of CrATP inhibition. The unexpectedly low V_{max} values for these two compounds may result from their failure to induce the conformation change necessary for catalysis or tight binding of CrATP.

TABLE I: Effect of Sugars on Degree of Inhibition by CrATP at 25°.[a]

Sugar (Concn, mM)	v_∞/v_0[b]	Half-time[c] (sec)
D-Fructose (1.4)	0.06	3
D-Mannose (0.1)	0.03	6
2-Deoxy-D-glucose (0.6)	0.12	5
D-Lyxose (8.33)	0.06	24
D-Glucosamine (1.5)	0.38	6
D-Arabinose (66)	0.66	120
D-Mannoheptulose (0.3)	0.80	16
1,5-Anhydro-D-mannitol (15)	*d*	*d*
1,5-Anhydro-D-glucitol (15)	*d*	*d*
2,5-Anhydro-D-mannitol (15)	*e*	*e*
2,5-Anhydro-D-mannose (2)	0.36	4
2,5-Anhydro-D-glucitol (92)[f]	<0.06	<45

[a] Hexokinase preincubated with sugar, 20 μM CrATP, and 200 μM MgATP for various times at 25°, and initial velocities determined by glucose-6-P dehydrogenase assay after addition of 10 mM glucose. [b] v_0 is initial velocity with no preincubation; v_∞ is initial velocity after preincubation for a long enough time for the change to be complete. [c] Time of preincubation required for initial velocity to change halfway from v_0 to v_∞. [d] No tightening observed. [e] Reaction was too rapid to see a change in the strength of CrATP inhibition. [f] Based on one sample incubated for 3 min.

Binding Studies. When [^{51}Cr]ATP was incubated at pH 7 and 25° for 10 min with 4 mg/ml of Boehringer hexokinase and 10 mM glucose, and the resulting mixture was placed on a Sephadex G-25 column, label was found in the protein peak as well as in the later band where CrATP is expected (Figure 5). Identical results were obtained whether 10 mM glucose was present in the eluting buffer (as in Figure 5) or not. With 350 μM CrATP, 6% of the material was bound, corresponding to 5.2 nmol/mg of hexokinase. With 35 μM CrATP, 25% of the CrATP was bound, but this was only 2.2 nmol/mg of hexokinase, suggesting either that all of the CrATP capable of combining had done so, or that binding was not complete because of the low level of CrATP. The former was shown to be the case by testing the ability of the residual CrATP in the second peak to inhibit hexokinase in a system containing 8 mM glucose and 100 μM MgATP. When fractions 16 and 17, and 20 and 27 of the 350 μM CrATP column shown in Figure 5 were tested (final concentrations 9.0 and 1.56 μM), the initial rate of the hexokinase reaction was inhibited 53 and 20%, which corresponds to an inhibition constant (after correction for MgATP) of 3–4 μM, as expected. When fractions 17 and 18 of the 35 μM CrATP column were used, however (final concentration 1.44 μM), no inhibition was observed, showing that that portion of the CrATP not binding to hexokinase under these conditions is not capable of inhibiting it.

The tight binding of CrATP induced by sugars could be caused either by a slow conformation change in the enzyme,

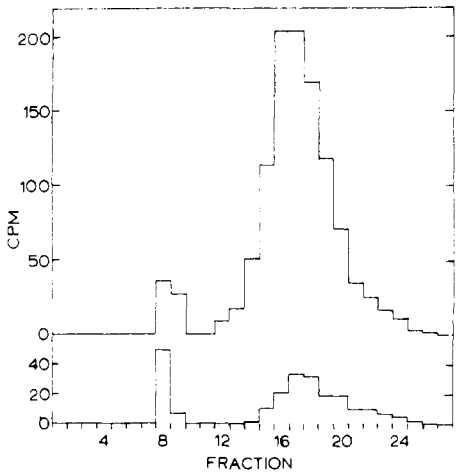


FIGURE 5: Binding of [^{51}Cr]ATP to hexokinase in the presence of glucose. (Top) Boehringer hexokinase (1 mg) was incubated with 350 μM [^{51}Cr]ATP (12,700 cpm/μmol), 10 mM glucose, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0) in 0.25-ml total volume for 10 min at room temperature. The mixture was then placed on a 1 × 20 cm column of Sephadex G-25 which contained 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7) and 10 mM glucose. Elution was with the same solution, and fractions were 1.2 ml. (Bottom) Same, except 2 mg of hexokinase in 0.5-ml volume and 35 μM [^{51}Cr]ATP.

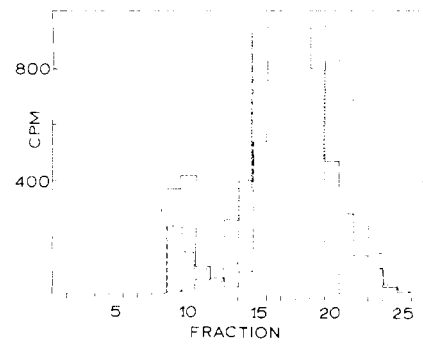


FIGURE 6: Binding of [^{14}C]glucose to hexokinase in the presence of CrATP or Cr$(NH_3)_4$-ATP. Sigma C-130 hexokinase (1 mg) was incubated in 0.25-ml volume with 1 mM [^{14}C]glucose (10^5 dpm/μmol), 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0) and nucleotide at room temperature for 10 min. The mixture was then placed on a 1 × 20 cm column of Sephadex G-25 and eluted with 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0). Fractions were 1.2 ml: (——) 350 μM CrATP; (- - -) 10 mM Cr$(NH_3)_4$-ATP; (• • •) no nucleotide. The protein emerged in fractions 9 and 10.

or by catalytic replacement of ligands on the chromium by ones from the enzyme. In the latter case, the chromium should become covalently linked to the enzyme, and denaturation of the resulting complex by cold perchloric acid should leave the chromium still bound to enzyme. To distinguish these possibilities the protein peak from an experiment similar to that shown in Figure 5 was split in equal parts, and to one part was added 0.1 vol of 60% $HClO_4$. After 1 hr at 4°, the denatured protein was removed by centrifugation and an aliquot of the supernatant and of the original solution were both counted. Both samples gave identical activities, showing that the chromium of CrATP does not become covalently attached to the enzyme during incubation with hexokinase and glucose.

When the binding studies were repeated with 350 μM unlabeled CrATP and 1 mM [^{14}C]glucose, labeled glucose was also found in the protein peak. The amount bound (2.2% of that added, or 5.4 nmol/mg of Boehringer hexokinase) corresponded to a 1:1 complex of CrATP and glucose, and was the same whether or not glucose was present in the eluting buffer. It thus appears that the hexokinase–CrATP–glucose complex, once formed, has a very long half-life and does not exchange with unbound molecules in solution at a rate that is appreciable over the time required to run this type of experiment.

The 1:1 nature of the complex was also confirmed by running parallel binding studies with Cr-[^{14}C]ATP and [^{14}C]glucose in a system containing 300 μM CrATP, 1 mM glucose, and 0.4 mg of Sigma type C-300 hexokinase in 0.5-ml, total volume. Of the Cr-[^{14}C]ATP, 6.6% was bound (27 nmol/mg of hexokinase), while of the [^{14}C]glucose, 2.1% was bound (26 nmol/mg). This degree of binding indicates a combining weight of 38,000 for this preparation of hexokinase (specific activity = 375 units/mg), as opposed to 190,000 for the Boehringer preparation (140 units/mg). This binding technique appears to be a useful and precise way to determine the purity of hexokinase preparations, although the combining weights given above are only approximate as they are based on the manufacturer's stated concentrations and activities for the enzyme preparations used.

The molecular weight of hexokinase has been reported to be 46,000 at pH 7 by Kenkare and Colowick (1965), and to vary from 42,000 to 48,000 by pH 7.6, depending on concentration, by Rudolph and Fromm (1970). It thus appears likely that one CrATP and one glucose combine per molecule of this size.

The tight binding of [^{14}C]glucose is induced not only by CrATP, but also by the weaker inhibitor, Cr$(NH_3)_4$ATP, as seen in Figure 6. In the absence of nucleotide, no glucose was seen in the protein peak, while 350 μM CrATP caused the binding of 11.6 nmol of glucose/mg of hexokinase, and 10 mM Cr$(NH_3)_4$ATP caused 3.7 nmol/mg to be bound. In the latter case, the lower yield suggests that the complex was decomposing slowly during chromatography. This is not unexpected, since Cr$(NH_3)_4$ATP might be expected to dissociate more rapidly because of its higher inhibition constant. The enzyme used in this experiment was Sigma C-130, 220 U/mg, and the combining weight with CrATP was calculated to be 86,000.

The binding of CrATP is induced not only by glucose, but also by other sugars that are good substrates or inhibitors of hexokinase. As shown in Table II, glucose, mannose, fructose, and 2,5-anhydroglucitol all induce the same degree of binding of CrATP. However, 2,5-anhydromannose, 2,5-anhydromannitol, glucose-6-P, and lyxose induce less binding, suggesting that the E–CrATP–sugar complex has partly decomposed during the chromatography. This is presumably caused by variation in the size of the rate constant for breakdown of the complex, but the fact that the E–CrATP–lyxose complex seems to break down faster than the others, while it forms more slowly (Table I), suggests that the sugars that form tight complexes change the dissociation constant of CrATP by a factor much larger than the factor of 40 measured for lyxose.

The reason for this result became obvious when the hexokinase–CrATP–[^{14}C]glucose complex isolated by Sephadex chromatography in 10 mM buffer was denatured at 4° by the addition of 0.02 ml of 60% $HClO_4$/ml of complex and by violent agitation in the presence of a drop of CCl_4. When the resulting filtered solution was diluted 1:10 and placed on a column of Dowex 50-H^+, 25% of the [^{14}C]glucose passed through the column and was not adsorbed on Dowex 1-Cl (presumably free sugar), while 75% was eluted in a focused band by 0.1 M lithium formate (pH 3.5) at the

TABLE II: Binding of Cr-[^{14}C]ATP to Hexokinase in the Presence of Sugars or Sugar Analogs.[a]

Sugar or Analog (Concn, mM)	CrATP Bound (μM)
None	0
D-Glucose (10)	75
D-Glucose (1)	84
D-Glucose (1[b])	26
D-Glucose (1[c])	52
D-Glucose (0.1)	67
D-Glucose (0.05)	39
D-Fructose (2)	72
D-Fructose (0.2)	88
D-Mannose (1)	83
2,5-Anhydro-D-glucitol (56)	80
2,5-Anhydro-D-glucitol (0.2)	21
2,5-Anhydro-D-mannose (2)	63
2,5-Anhydro-D-mannitol (15)	59
D-Glucosamine (15)	33
1,5-Anhydro-D-glucitol (15)	22
1,5-Anhydro-D-glucitol (1)	4
1,5-Anhydro-D-mannitol (15)	3
2,5-Anhydro-D-lyxitol (100)	8
1-Deoxy-D-fructose (10)	15
D-Glucose-6-P (10)	46
D-Glucose-6-P (1)	14
6-Deoxy-D-glucose (10)	24
D-Lyxose (10)	35

[a] Experiments were similar to those in Figure 6 with 1.2 mg of Sigma F-300 hexokinase (250 units/mg) and Cr-[^{14}C]ATP (48,500 cpm/μmol), 460 μM. [b] Cr-[^{14}C]ATP, 115 μM. [c] Cr-[^{14}C]ATP (210,000 cpm/μmol) prepared by heating for only 2 min at 80°, 110 μM.

point where the pH of the eluate changed from 2.6 to 3.5. This is the elution position predicted for the doubly monodentate CrADP–glucose-6-P complex which would result if phosphate were transferred from $\beta\gamma$-bidentate CrATP to glucose without breaking the coordination bonds to chromium. This complex is a slow substrate for glucose-6-P dehydrogenase (V_{max} about 1% that of glucose-6-P) and upon heating with EDTA liberates free glucose-6-P and ADP. The formation of CrADP–sugar-6-P complexes from Sephadex-prepared hexokinase–CrATP–sugar complexes has also been demonstrated for fructose (87%), mannose (92%), glucosamine (50%), 2,5-anhydromannose (54%), 2,5-anhydromannitol (58%), 2,5-anhydroglucitol (55%), and with glucose and $Cr(NH_3)_4ATP$ (25%), and glucose and Cr–8-BrATP (80%). The observed percentage of CrADP–sugar-6-P complex in these experiments appears to represent the phosphorylation equilibrium on the enzyme, since the rate of formation of this complex appears to parallel the conformation change that leads to tight binding of CrATP. When freshly prepared CrADP–mannose-6-P was tested as an inhibitor of hexokinase, it had a K_i of about 3 μM, but showed little change in inhibition with time (the change which did occur was explainable on the basis of the 8% of CrATP that should have also been released when the protein was denatured). The CrADP–glucose-6-P complex is also slowly released from the enzyme with a rate constant of about 0.04 min^{-1}, so it appears from these experiments

TABLE III: Inhibition of Hexokinase by Chromium Complexes.

Inhibitor (Concn, μM)	Initial K_i[a] (μM)	Final K_i[a] (μM)
CrATP (10)	4.9	0.18
$Cr(NH_3)_2ATP$ (10)	9.2	0.4
CrATP (10)	5	0.4
$Cr(NH_3)_3ATP$ (100)	60	4
$Cr(NH_3)_3ATP$ (50)	62	14
$Cr(NH_3)_3ATP$ (10)	50	*b*
$Cr(NH_3)_4ATP$ (*c*)	150[d]	12
$Cr(NH_3)_4ATP$ (*c*)	420[e]	50
$Cr(NH_3)_4(H_2O)ATP$ (*c*)	1300[e]	*b*
$Cr(NH_3)_5ATP$ (*c*)	3200[d]	*b*
Cr–adenosine tetraphosphate (100)	60	*b*
Cr–adenylyl imidodiphosphate (900)	300	*b*
$Cr(NH_3)_4$–adenylylimidodiphosphate (630)	2300	*b*
Cr–8-BrATP (0.92)	0.14	0.01
$Cr(NH_3)_4$–8-BrATP (100–1000)	210	20
CrADP–P_i (130, 390)	165	*b*
CrAMP (400, 800)	1200	*b*
Monodentate CrADP (170–1250)	100	20
CrADP (2000)	2000	*b*
$Cr(NH_3)_2ADP$ (2000)	1300	*b*
$Cr(NH_3)_4ADP$ (2000)	9000	*b*

[a] It was assumed that the inhibitions were competitive *vs.* MgATP, and the kinetics followed eq 1. By comparing velocity in the absence of inhibitor (v_0) with that in the presence of inhibitor (v_i), K_i is calculated from $K_i = I/[(1 + A/K_a)\cdot((v_0/v_i) - 1)]$, where I is inhibitor concentration, A is MgATP concentration (100 μM), and K_a its apparent K_m (100 μM). Initial K_i was calculated from initial velocity. Final K_i, when given, was calculated from rate seen after several minutes when the lower steady-state rate appeared to have become established. [b] The K_i did not appear to change over several minutes. [c] Apparent K_i value from a $1/v$ *vs.* I plot divided by $(1 + A/K_a)$. These experiments were carried out at 25° by M. I. Schimerlik. [d] From heating $[Cr(NH_3)_5(H_2O)](NO_3)_3\cdot NH_4NO_3$ with ATP. [e] From heating $[Cr(NH_3)_4Cl(H_2O)]Cl_2$ with ATP.

(which are continuing and will be reported in detail later) that CrATP is an actual substrate for hexokinase, but with a very low V_{max}.

Specificity for CrATP. In their studies on the inhibition of six kinases by different chromium nucleotides, Janson and Cleland (1974b) have shown that only CrATP is a good inhibitor for yeast hexokinase, and that replacement of adenine by other bases drastically raises the K_i and eliminates the tightening of inhibition induced by the sugar. Chromium nucleoside diphosphates were poor inhibitors, and the only compounds to show the tightening phenomenon other than CrATP were $Cr(NH_3)_2ATP$ and $Cr(NH_3)_3ATP$. However, the replacement of coordinated water by NH_3 raised the K_i both for initial velocities and for the final velocities seen after the tightening has taken place. Table III shows this phenomenon in more detail and also includes the chromium complexes of a number of other adenine nucleoside derivatives. In addition to the compounds shown, chromium-α,β-methylene ATP and chromium-$\beta\gamma$-methylene

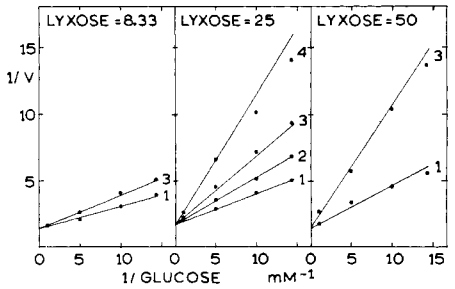


FIGURE 7: Competitive substrate inhibition by MgATP induced by the presence of lyxose; MgATP: (1) 0.5 mM; (2) 2 mM; (3) 4 mM; (4) 10 mM. Lyxose concentrations in millimolar units. Each pattern fitted separately to eq 1.

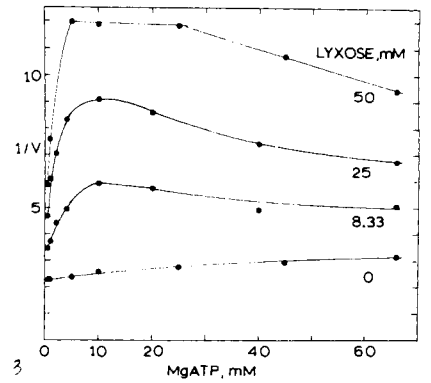


FIGURE 8: Partial substrate inhibition by MgATP induced by lyxose; glucose, 100 μM.

ATP did not inhibit at 100 μM, and $Cr(NH_3)_4PP$ and $Cr(NH_3)_5PP$ did not inhibit at 700 μM.

Lyxose-Induced Substrate Inhibition by MgATP. In order to better understand the effects of lyxose on CrATP binding, this induced substrate inhibition was studied at three lyxose levels by varying glucose at high fixed MgATP concentrations (Figure 7). The inhibitions appear to be linear competitive, and the data were fitted to eq 1. When higher levels of MgATP were used, however, the substrate inhibition was clearly partial (Figure 8), and reached its peak at or below 10 mM MgATP. The inhibition was slightly overcome at still higher MgATP, probably because of effects of the higher ionic strength on the binding of MgATP or lyxose (the apparent K_m values of glucose and MgATP are raised by factors of 22 and 36 in 1.5 M NaCl (Rudolph and Fromm, 1970)).

The inhibition of lyxose *vs.* glucose is competitive, but the K_{is} varies from 4.2 mM at 10 mM MgATP to 80 mM at 0.1 mM MgATP (data not shown). When MgATP was varied at concentrations below the substrate inhibition level (Figure 9), lyxose was a noncompetitive inhibitor with K_{is} = 500 ± 80 mM and K_{ii} = 79 ± 4 mM from a fit to eq 2.

Discussion

The clearly noncompetitive inhibition of CrATP *vs.* glucose (Figure 1) provides strong evidence that the kinetic mechanism of yeast hexokinase is random, and not ordered with glucose adding first. If CrATP could only add after glucose, the inhibition *vs.* glucose would be uncompetitive, but in fact CrATP appears to combine with free enzyme equally well as with E-glucose.

The inhibition by CrATP tells us much more about the mechanism than just that it is random, however. First, the dissociation constant of the active isomers of CrATP is

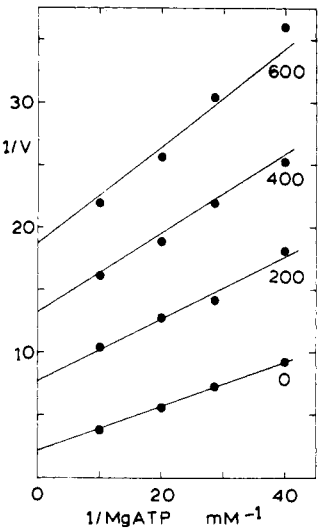
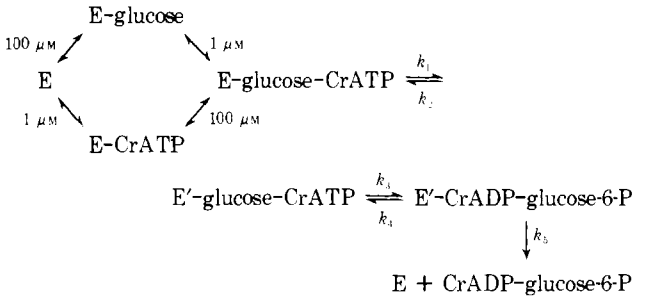


FIGURE 9: Noncompetitive inhibition of lyxose vs. MgATP: glucose, 100 μM; lyxose levels are in millimolar units.

100-fold lower than the K_m of MgATP (and as noted below, it is 5000-fold lower than the K_i for MgATP), so that it can be considered a transition state analog. The inhibition is, however, freely reversible (first entry, Table II). Second, upon incubation with a sugar that is a substrate or inhibitor of the enzyme, the dissociation constant is further decreased by a factor as high as 40, and the resulting complexes break down slowly. Finally, with sugars that are good substrates, phosphate transfer occurs to give a CrADP-sugar-6-P complex in equilibrium with CrATP and free sugar on the enzyme. CrADP-sugar-6-P, where the ADP and sugar-6-P are held together by their inert coordination bonds to the chromium, can be released very slowly from the enzyme (0.04 min^{-1} for CrADP-glucose-6-P), making CrATP a substrate with an extremely low V_{max}. With inhibitors or poor substrates where this change does not occur, the tight E-sugar-CrATP complexes decompose more readily during Sephadex chromatography as one would predict.

The simplest model which appears to account for the data is



where k_1/k_2 is about 40, and k_1 is between 1 and 2 min^{-1} at 17°, based on the observed half-times for the tightening of inhibition by CrATP (the value of 100 μM for the dissociation constant of glucose is taken from the kinetic model described later in the discussion). The value of k_3 is probably greater than that for k_1, since the yield of CrADP-glucose-6-P upon acid denaturation seems to parallel the formation of the tighter E′-glucose-CrATP complex, and the ratio of k_3/k_4 is 3, based on the 75% yield of CrADP-glucose-6-P after denaturation of the enzyme. The constant k_5 is about 0.04 min^{-1}, the same size as k_2. The partial decomposition of the E′-glucose-$Cr(NH_3)_4$ATP complex

during Sephadex chromatography probably reflects the higher rate constant for release of $Cr(NH_3)_4ATP$ from E-glucose-$Cr(NH_3)_4ATP$, and the 25% yield of $Cr(NH_3)_4ADP$-glucose-6-P upon acid denaturation of the remaining complex suggests that k_3/k_4 is 0.33 in this case.

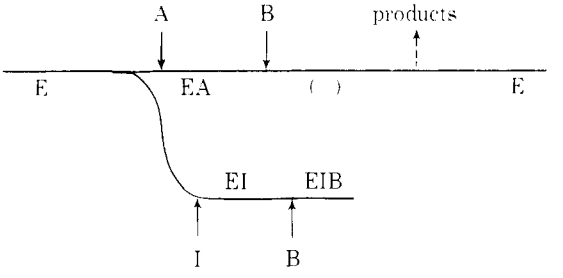
Coordination State and Conformation of the Nucleotide Substrate. The data from the experiment shown in Figure 5 suggest that only 25% of the CrATP was capable of forming the tight E′-glucose-CrATP complex, and that the other 75% was not inhibitory in the hexokinase reaction. The third line in Table II shows that 22% of this CrATP preparation was bound in the presence of excess enzyme. This shows that the reaction is a specific one, and raises the question of which CrATP isomers are the ones inhibiting the reaction and forming the E′-glucose-CrATP complex. Two β,γ-bidentate, and four α,β,γ-tridentate isomers of CrATP are possible,[2] and recent work in this laboratory by Dr. D. O. Brummond suggests that all six exist, with the tridentate forms predominating. Interconversion between these isomers is very slow, and the isomeric content of various preparations does not appear to change over several months. Furthermore, it appears that it is the bidentate isomers only that inhibit yeast hexokinase (Brummond and Cleland, 1974). In agreement with this postulate, 48% of Cr-[^{14}C]ATP prepared by heating for only 2 min at 80° is bound in the presence of excess hexokinase (line 4, Table II). During the heating process γ-monodentate CrATP forms first, followed by bi- and then an equilibrium mixture of bi- and tridentate forms. This is consistent with the good inhibition and tightening with the time seen with the bidentate $Cr(NH_3)_4ATP$ complex, but poor inhibition by the monodentate $Cr(NH_3)_4(H_2O)ATP$ and $Cr(NH_3)_5ATP$ complexes (Table III). $Cr(NH_3)_2ATP$ and $Cr(NH_3)_3ATP$ exist as both bidentate and tridentate isomers, with about 30% being bidentate. The inhibition by chromium-adenosine tetraphosphate may reflect the similarity of the β,γ,δ-tridentate isomers of this complex with the β,γ-bidentate isomers of CrATP, although the tightening phenomenon is not seen. The CrADP-P_i complex is a surprisingly good inhibitor, although again the tightening of inhibition is not seen. The chromium-methylene ATP complexes are poor inhibitors, but they do not inhibit creatine kinase either, while CrADP-P_i and chromium-adenosine tetraphosphate do (apparent K_i values of 190 and 220 μM, compared to 100 μM for CrATP). The surprising compounds are chromium-adenylyl imidodiphosphate and the corresponding tetraammine. The aquo complex is as good an inhibitor of creatine kinase (apparent K_i, 110 μM) as CrATP, and the tetraammine has to be bidentate, so the failure of these compounds to inhibit hexokinase suggests a high degree of specificity for the nature of the linkage between the β- and γ-phosphates. Possibly the greater positive charge that will reside on the NH bridge in these compounds than on the oxygen bridge in CrATP is responsible for the effect.

If the active substrate for hexokinase is the β,γ-bidentate MgATP complex, as suggested above, the product of the reaction should be β-monodentate MgADP, and it would be clear why CrADP, which is an α,β-bidentate complex, is such a poor inhibitor. However, β-monodentate CrADP should be a good inhibitor of hexokinase, and might undergo the tightening seen with CrATP. When CrADP was prepared by heating a solution 20 mM each in NaADP and $CrCl_3$ at 80° for only 1 min rather than the usual 10 min, the resulting product showed an initial K_i of 100 μM, and a K_i of 20 μM after the tightening had occurred (Table III). It thus appears that monodentate CrADP does behave in a similar manner to bidentate CrATP, although the K_i values are somewhat higher and the change in strength of binding of sugars somewhat less.

One more fact can be deduced about the geometry at the hexokinase active site from the fact that Cr 8-BrATP is a better inhibitor than CrATP, and the tetrammines are equivalent. 8-BrATP exists largely in the syn as opposed to the anti conformation favored for ATP, and these data suggest that ATP is adsorbed to hexokinase in the syn conformation, or something close to it.

The Effects of Lyxose. Lyxose, along with other sugars that are good substrates or inhibitors of hexokinase, induces tighter binding of CrATP. It also causes a 40-fold drop in the apparent K_m (which should equal the dissociation constant) of MgATP for the slow ATPase reaction catalyzed by hexokinase (DelaFuente *et al.*, 1970). Lyxose is a competitive inhibitor *vs.* glucose, but the K_i changes from 4.2 to 80 mM as MgATP is lowered from 10 to 0.1 mM, suggesting that MgATP must be present for lyxose to bind. Conversely, when the compounds ATP-(6-glucose) and adenosine tetraphosphate-(6-glucose) were recently synthesized as possible transition state analogs by Dr. Peter Danenberg, they were competitive *vs.* MgATP, but uncompetitive *vs.* glucose, suggesting that they were acting as MgATP analogs and bound only in the presence of glucose.[3] These data can all be explained by assuming that sugars and nucleotides bind 40-fold tighter when both are present, than when only one has combined with the enzyme. The substrate inhibition by MgATP induced by the presence of lyxose can also be explained on this basis, and since this induced substrate inhibition is important for an understanding of the hexokinase mechanism, it will be analyzed in detail.

Fromm (1967) first pointed out that in an ordered mechanism where a dead-end inhibitor combined with free enzyme only, that if the second substrate could add to the EI complex, substrate inhibition by the second substrate competitive *vs.* the first was induced by the presence of the dead-end inhibitor.



(3)

Thus in mechanism 3, B will show substrate inhibition competitive *vs.* A when I is present, and the higher the level of I, the stronger the inhibition. Since other kinetic evidence

[2] The γ-monodentate CrATP complex has been recently prepared in this laboratory and shown to have a higher pK for the secondary phosphate group than the bi- and tridentate complexes. It was the precursor seen at short heating times by DePamphilis and Cleland (1973), and mistakenly postulated to be a bidentate complex; it is removed during purification of CrATP.

[3] If the dissociation constant were 40-fold higher in the absence of glucose, the slope effect in these patterns would be just below the level for detection, and the patterns would still look uncompetitive *vs.* glucose. In these molecules, the glucose apparently hangs out in solution and is not bound; their K_i values are about 200 μM.

pointed to a random mechanism for yeast hexokinase, Rudolph and Fromm (1971a) first attributed the induced substrate inhibition caused by lyxose to combination of MgATP at two sites on the enzyme, but later suggested that lyxose induced a conformation change that persisted after replacement of lyxose with glucose, and that in the modified conformation, certain rate constants had values that led to substrate inhibition by MgATP (Rudolph and Fromm, 1971b). These authors failed to realize, however, that the substrate inhibition induced by lyxose was partial (Figure 8), and that partial induced substrate inhibition may occur in random mechanisms.

The substrate inhibition in mechanism 3 is caused by buildup of the EIB complex at high B levels, and this complex becomes the only enzyme form present at infinite B, since the E-EI-EIB sequence is at equilibrium, while the E-EA-(EAB) sequence is not because of the conversion of (EAB) to products. In a random mechanism, combination with EB only (or at least much tighter binding to EB than to E) of a dead-end inhibitor competitive with A will lead to large levels of EIB when B is infinite. If at the same time the steady-state level of EB is small when both A and B are at K_m levels, then the inhibitor will induce partial substrate inhibition by B. The substrate inhibition is of course competitive *vs.* A, since A and I compete for the EB form, and the severity of the inhibition depends on I in a hyperbolic fashion, rather than the linear manner which results from mechanism 3. In order for the steady-state level of EB to be low at levels of A and B equal to K_a and K_b, the mechanism cannot be of the rapid equilibrium type, the K_m for B must be considerably below its actual dissociation constant from the enzyme, and the majority of the reaction flux at K_m levels of the substrates must follow the path where A adds before B. (At levels of B close to its dissociation constant, of course, more flux goes through the path where B adds first. If the physiological level of B is closer to its dissociation constant than to its Michaelis constant, there will not be a preferred path under physiological conditions.) In practice this means the rate constant for release of A from (EAB) must be smaller than V_{max}, and considerably smaller than that for breakdown of EA.

In such a mechanism the ratio of the rates in the absence (v_0) and presence (v_i) of lyxose when MgATP is at infinite concentration is given by

$$\frac{v_0}{v_i} = 1 + \frac{I}{K_I(1 + A/K_a)}$$

where A is glucose and K_a its K_m, I is lyxose, and K_I its dissociation constant from E-MgATP. With the data in Figure 8 for 10 mM MgATP it is then possible to estimate the value of K_I for lyxose as 3-6 mM, in agreement with the K_i of 5 mM reported by DelaFuente *et al.* (1970), and the value of 4.2 mM measured in this laboratory at 10 mM MgATP. The dissociation constant of lyxose from free enzyme is much higher; the noncompetitive inhibition *vs.* MgATP (Figure 9) gives a value of 240 ± 40 mM.[4]

Comparison of Reaction with Chromium and Magnesium Nucleotides. When both sugar and nucleotide are present, something happens which leads to an apparent increase in binding of at least 40-fold. The change is very fast with Mg nucleotides, but slow enough to follow easily with Cr nucleotides. Since CrATP has equal affinity initially for the enzyme in the presence or absence of glucose, it would appear that a conformational change in the protein occurs which leads to tighter binding of substrates and substrate analogs.[5] Presumably this conformation change is a prelude to the actual catalytic process itself. The reason for the slowness of the change with Cr nucleotides is not apparent, unless it involves some changes in the coordination sphere of chromium. Since chromium does not become bound to the protein, however, it is not clear what changes could be involved. The half-time of the change is not appreciably different for CrATP and $Cr(NH_3)_4ATP$, and since NH_3 is much more difficult to displace from chromium than water, this argues against ligand substitutions during the change. Since the conformation change is equally slow whether an inhibitor like lyxose or a substrate like glucose is present, the ability to undergo phosphorylation is not involved. Possibly the rigidity of the Cr nucleotides is responsible (for example, if deformation of the normal coordination octahedron toward a trigonal prism occurred during the conformation change, the change would be expected to be much slower with Cr than with Mg), but whatever the reason, the possibility of studying this conformation change on a more convenient time scale than that which would be required with Mg nucleotides is certainly very exciting.

The subsequent chemical changes which occur in hexokinase-sugar-CrATP complexes with sugars that are substrates are equally exciting, since it appears that CrATP is acting as a slow substrate. An equilibrium between CrATP and glucose and the CrADP-glucose-6-P complex seems to be established as rapidly as the conformation change that causes tight binding, and CrADP-glucose-6-P is then released at a rate of about 0.04 min^{-1}. Since the CrADP-mannose-6-P complex has a K_i of about 3 μM, it is probable that the slow rate of release is caused by the slow rate of a conformation change analogous to the one that converts the loose E-glucose-CrATP complex into the tight one capable of catalytic reaction. The lack of observed tightening when CrADP-glucose-6-P is used as an inhibitor is consistent with an equilibrium constant for this change of near unity, unlike the value of 40 seen with CrATP and glucose. The similarities between this model of CrATP activity and that derived for MgATP in the following section (except for the drastically different time scale of the conformation changes,

[4] The slope effect is caused solely by the combination of lyxose with free enzyme but the model presented in mechanism 5 predicts that K_{is} will be 2.1 times the dissociation constant of E-lyxose. K_{ii} depends on the dissociation constants for lyxose from both free enzyme and E-MgATP, although more heavily on the latter, but again the exact relationship depends on the constants assumed in mechanism 5. Assuming mechanism 5, the dissociation constant of lyxose from E-MgATP can be calculated from K_{ii} as 1 mM. By comparing mechanism 5 with the K_{is} values from the competitive inhibition of lyxose *vs.* glucose, dissociation constants for lyxose from E-MgATP are calculated as 2.1 or 2.8 mM. The reasonably good agreement here, despite the fact that the constants in mechanism 5 were not specifically picked to produce this result, gives some confidence in the final kinetic model presented below.

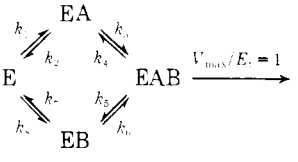
[5] It is important that the conformation change occurs only after binding of both substrates, as shown by the equal initial affinity of CrATP for E and E-glucose. With glycerokinase from *Canida mycoderma*, which is also inhibited strongly by β,γ-bidentate CrATP (K_i = 0.5 μM; Janson and Cleland, 1974a), combination of glycerol and MgATP occurs only in that order, and CrATP binds only to E-glycerol, and not to E or E-glycerol-P. In ordered mechanisms, the conformation change which permits catalysis presumably takes place after binding of the first substrate rather than after both substrates combine, and that may be what makes the mechanism ordered, rather than random. Such an explanation is consistent with the observation that isomerization of binary complexes is the rule in ordered mechanisms (that is, the form resulting from addition of the first substrate must undergo isomerization before the second can add).

and the much tighter binding of reactants) will become apparent.

A Kinetic Model of the Hexokinase Reaction with MgATP and Glucose as Substrates. By means of pulse chase experiments, Rose *et al.* (1974) have recently determined the rate constant for breakdown of E-glucose to be 30% of V_{max}, and the constant for release of glucose from E-glucose-MgATP to be less than 5% of V_{max}. These experiments were carried out with high levels of enzyme that had been treated with trypsin to improve its affinity for glucose, and since Rudolph and Fromm (1970) believe that the kinetic properties of hexokinase change upon dilution, the values may be somewhat different with dilute enzyme not treated with trypsin. However, they do indicate that glucose is released very slowly from the E-glucose-MgATP complex, as is required to permit induced substrate inhibition by MgATP in the presence of lyxose.

If one takes these data, the K_m values observed in the present work (100 μM for both glucose and MgATP), and the available values for the dissociation constants of E-glucose and E-MgATP, one can construct a kinetic model of hexokinase that is consistent with all of the experimental data available. Rudolph and Fromm (1971b) attempted this, but lacking the data of Rose *et al.* (1974) they assumed that glucose release from E-glucose was much faster than V/E_t. Zewe *et al.* (1964) determined the dissociation constant of glucose at pH 7.6 as 144 μM by fluorescence titrations with 0.1 mg/ml of enzyme. Colowick and Womack (1969) found 32 μM for glucose at pH 7 with 10 mg/ml of enzyme, and Rose *et al.* (1974) found 23 μM under identical conditions except with trypsin-treated enzyme. Noat *et al.* (1969) reported 100 μM for glucose at pH 8.6 (identical with K_m), but could not detect MgATP binding (K_i above 1.2 mM). For the model discussed below we will choose 100 μM for the dissociation constant of glucose. The apparent K_m of MgATP for the very slow ATPase reaction catalyzed by hexokinase should be the dissociation constant and is 5 mM at pH 8 (Kaji and Colowick, 1965) and 4–10 mM at pH 8.6 (Noat *et al.*, 1969).

In addition to being consistent with the above data, any hexokinase model must also produce initial velocity patterns for MgATP and glucose with linear reciprocal plots that cross on the horizontal axis. This is a serious restraint, since as Cleland and Wratten (1970) have pointed out, unless the unimolecular rate constants (k_2, k_4, k_5,k_7) exceed V_{max}/E_t, a random mechanism such as that in mechanism 4 will



(4)

A = MgATP $K_a = (1 + k_5)/k_6$

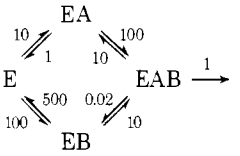
B = glucose $K_b = (1 + k_4)/k_3$

give linear reciprocal plots only if $k_2 = k_7$, $k_8/k_3 = k_5/(k_4 + k_5)$, $k_1/k_6 = k_4/(k_4 + k_5)$, and thus $k_8/k_3 + k_1/k_6 = 1$. Alternatively, if $k_4 > V_{max}$, and $k_5 < V_{max}$, the criterion $k_1 = k_6$ is sufficient and k_7 may be considerably greater than k_2. Since k_5 is much less than V_{max}/E_t and k_2 the same size or smaller (Rose *et al.*, 1974), these criteria must be met rather closely, or the reciprocal plots will be noticeably nonlinear. Further, if the pattern is to cross on the horizontal axis

$$k_2 = \frac{(1 + k_5)(1 + k_4)}{(1 + k_4 + k_5)}$$

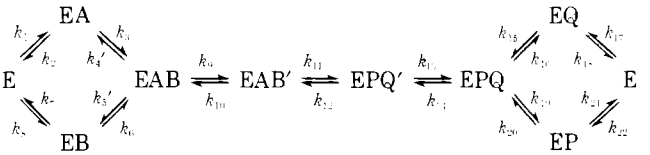
which, since k_5 is small, effectively makes $k_2 = 1 = V_{max}/E_t$. The lower value seen by Rose *et al.* (1974) may reflect the effect of trypsin treatment (it would be of interest to see if reciprocal plots become nonlinear for trypsin-treated enzyme.)

When $k_2 = k_7 = V_{max}/E_t$ in mechanism 4, the initial velocity patterns are linear and cross on the horizontal axis, but reciprocal plots *vs.* glucose at high MgATP in the presence of lyxose (assuming lyxose to combine only with E-MgATP) become noticeably curved, unlike those in Figure 7. When $k_1 = k_6$, k_4 is made large, and k_7 is larger than k_2, however, a model is obtained which not only produces the correct initial velocity patterns, but also gives linear plots *vs.* glucose at high MgATP in the presence of lyxose. In this model (mechanism 5), unimolecular constants have values relative to V_{max}/E_t, and bimolecular ones are picked to give the proper K_m values in millimolar units.[6] The bimolecular constants are also assumed not to depend on the presence of the other substrate on the enzyme.



(5)

However, the model shown in mechanisms 4 and 5 does not account for all of the information available for hexokinase, and in order to consider the kinetics of the back reaction and to account for the 40-fold tighter binding of sugars and nucleotides once both are present, a more complete and realistic model such as that in mechanism 6 is needed. In



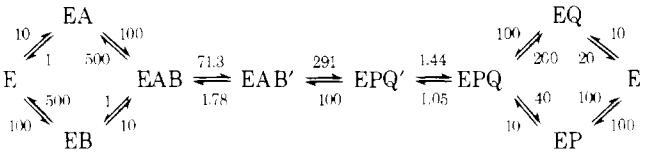
A = glucose
B = MgATP
P = MgADP
Q = glucose-6-P

(6)

this model the initial EAB complex changes into the activated EAB′ form prior to catalysis, and similarly, EPQ′ must change to EPQ before products can be released. If the

[6] The model given here is of course not completely unique, and k_3 and k_4 or k_7 and k_8 can be increased or decreased together in constant ratio, as long as $k_4 > V/E_t$, and $k_7 > k_2$. The values of k_1, k_2, and k_6 must be close to those shown, however, if reciprocal plots are to be linear and cross on the horizontal axis. The value of k_5 is picked to make $K_{i,MgATP}$ = 5 mM. If k_5 = 0.1 and k_7 = 100, $K_{i,MgATP}$ becomes 1 mM, but it is not possible to get a lower value without accepting a higher value of k_5, in conflict with the results of Rose *et al.* (1974). Thus, the observed $K_{i,MgATP}$ values around 5 mM are probably correct. The modeling studies described here were carried out with a computer program that, when given the rate constants in mechanism 4, calculates for a grid of A and B concentrations the reciprocal velocity, proportion of enzyme in E, EA, EB, EAB, and the proportion of reaction flux going *via* the paths involving EA and EB. The presence of induced substrate inhibition is also checked for by calculating at each point the reciprocal velocity if a dead-end inhibitor combines with EB only. These studies show that the criteria of Cleland and Wratten (1970) must be closely adhered to if linear plots crossing on the horizontal axis are to be obtained, and thus essentially establish the validity of mechanism 5 as a description of the initial velocity kinetics of hexokinase.

unimolecular constants are chosen so that $V_1/E_t = 1$, then k_1, k_2, k_3, k_6, k_7, and k_8 are identical in mechanisms 4 and 6, but k_4' and k_5' will be larger than k_4 and k_5. When the following assumptions are made, we obtain the unique model shown in mechanism 7:[7] (1) $k_9/k_{10} = 40$; (2) $k_4' = k_7$ and $k_5' = k_2$, so that the actual dissociation constant for nucleotide is unaffected by the presence of sugar and *vice versa* (as seen for CrATP before the tightening takes place). (3) V_1 is about 7 times V_2. (At pH 6.5, $V_1/V_2 = 2.2$ (Fromm *et al.*, 1964), and since the K_m values seem not to change greatly with pH, this ratio should be 3.16 times larger at pH 7. The ratio of 15 seen by DelaFuente and Sols (1970) at pH 7 is too large because the assay used in the reverse direction detects β-glucopyranose only, and the mutarotation of glucose is slower than the enzymatic reaction.) (4) The initial velocity pattern in the reverse direction crosses on the horizontal axis (DelaFuente and Sols, 1970), and the K_m values are 2 mM for glucose-6-P and 1 mM for MgADP (based on the values observed in this laboratory, but in general agreement with those reported by Fromm *et al.* (1964) and DelaFuente and Sols (1970)). (5) The bimolecular constants for combination with the enzyme are the same for glucose and glucose-6-P, and the same is true for MgATP and MgADP. Further, the constants do not depend on whether the other substrate is present. (6) The binding of glucose-6-P and MgADP is slightly weaker when both are present. (Since the rapid equilibrium assumption holds in the reverse direction,[8] this assumption is necessary if the initial velocity pattern is to cross on the horizontal axis. A factor of 2 was arbitrarily chosen. The effect is reasonable, since both molecules are negatively charged.) (7) k_{11} and k_{12} are both much larger than k_{10} and k_{13}. (That is, catalysis is not rate limiting; k_{12} was arbitrarily set equal to 100, and the value of k_{11} determined). (8) $k_{16} > k_{19}$ in agreement with the results of Kosow and Rose (1970). (9) The constants in mechanism 5 are a valid starting point.



(7)

By changing these assumptions, slightly different rate constants are obtained, but the basic elements of the model are the same. This model produces $V_{max} = 1$ in the forward direction, and 0.143 in the reverse reaction. Note that k_4' and k_5' in mechanisms 6 and 7 are 50 times the values of k_4 and k_5 in mechanisms 4 and 5. The apparent low value of k_5 seen by Rose *et al.* (1974) and the K_m of MgATP that is 2% of the dissociation constant are thus the result of the ratio of 40 between k_9 and k_{10}. The observation of appreciable binding by dead-end inhibitors such as lyxose and ATP-(6-glucose) only when the complementary substrate is present is explained by the fact that these inhibitors mimic the substrate they replace well enough to permit the conformation change represented by k_9 to take place. Although mechanisms 6 and 7 are random, the disparity in the values of k_4' and k_5' results in the upper pathway *via* EA carrying 98% of the reaction flux when both substrates are present at K_m levels, in general agreement with previous isotope exchange studies which indicated that this was the predominate pathway (Fromm *et al.*, 1964; Britton and Clarke, 1972), and the induced substrate inhibition by MgATP in the presence of lyxose. However, at physiological MgATP, which is closer to the K_i value of 5 mM than to the K_m, the two pathways would carry nearly equal flux, especially if glucose were below its K_m.

In their analysis of the kinetics of phosphofructokinase, Bar-Tana and Cleland (1974) showed that when both substrates in the faster direction of a random mechanism dissociated more slowly than V_{max}, the initial velocity patterns were distorted so that they appeared nearly parallel, thus causing confusion with ping-pong mechanisms. With hexokinase, only one of the two substrates (glucose) dissociates more slowly than or at the same rate as V_{max}, and this fact, plus the high equilibrium constant for the conformation change that occurs as soon as both substrates are present, leads to the normal intersecting initial velocity pattern, K_m for MgATP that is far below its true dissociation constant, and the resulting confusion with an ordered mechanism. It cannot be overemphasized that kinetic studies need to be thorough and consist of a number of different types of experiment that will independently corroborate each other. The mistakes that have been made in the past in dealing with kinases such as hexokinase and phosphofructokinase should be a lesson to all.

[7] The values in mechanism 7 are derived by solving simultaneously the four equations for V_1, for V_2, for the ratio between k_4 and k_4', and for the analogous ratio (assumed to be 2) between the values of k_{16} in mechanism 6 and in a simplified scheme similar to mechanism 5, with k_{10}, the ratio of k_{11} and k_{12}, k_{13} and k_{14} as the unknowns. With the other assumptions noted, this gives a unique solution as shown, which is well determined and not very sensitive to changes in the assumptions. The most uncertain of the assumptions is the value of 40 for k_9/k_{10}, which is based on the observed effects of lyxose on the ATPase reaction with MgATP as substrate, and on the inhibition of CrATP. This value may be different for glucose and lyxose, but the apparently uncompetitive inhibition of ATP-(6-glucose) *vs.* glucose requires a value of at least 40, and values very far above 40 seem unlikely, and give ratios of k_{11}/k_{12} below unity. Although the agreement is certainly partly fortuitous, note that the equilibrium constant for the actual catalytic conversion of EAB' to EPQ' in mechanism 7, 2.91, is the same as that observed experimentally for CrATP and glucose (3.0). Thus, for both Mg and Cr nucleotides, the free energy is dissipated largely during the binding and conformation change steps, rather than during catalysis, and in the reverse reaction considerable free glucose and nucleotide would be present on the enzyme, despite the unfavorable overall equilibrium for the reaction. The value of 1280 for K_{eq} calculated from the constants in mechanism 7 is consistent with the value of 1550 ± 290 calculated for pH 7 by Robbins and Boyer (1957), and the one value actually measured at pH 7 by these workers of 1310. The model could be made to fit either of these values exactly by small changes in the K_m values of the reactants, but only small changes would occur in the rate constants.

[8] For a random mechanism the rapid equilibrium assumption must always hold in the direction with the lowest V_{max}, since the substrates must dissociate from the enzyme at rates greater than or equal to the faster V_{max} in the other direction.

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Studies of Specificity of Deoxyribonuclease from Salmon Testes†

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ABSTRACT: A limited digest of thymus DNA with salmon testes DNase was composed of fragments larger than tetranucleotides. With exhaustive digestion mono-, di-, and trinucleotides were formed. At the (3′) ω terminus G predominated, its frequency ranged from approximately 35 to 72% and increased with decreasing size of fragments. The error caused by the ribose-containing contaminants of DNA is significant, and should not be neglected in the evaluation of nucleoside frequency at the terminal positions of fragments.

A highly potent DNase was isolated from testes of mature salmon, *Oncorhynchus tshawytscha* (Yamamoto, 1971; Yamamoto and Bicknell, 1972). The enzyme was optimally active in the acid pH range, hydrolyzed calf thymus DNA endonucleolytically to yield oligomers bearing 3′-terminal phosphate, did not attack RNA, required no divalent cations, and cleaved native DNA ten times faster than denatured DNA. On the basis of these studies salmon testes DNase was characterized as a DNase II like enzyme (Yamamoto and Bicknell, 1972).

Several laboratories have investigated the specificity of DNase II like enzymes of spleen (Koerner and Sinsheimer, 1957; Doskocil and Sorm, 1961; Vanecko and Laskowski, 1962; Carrara and Bernardi, 1968; Ehrlich *et al.*, 1971; Devillers-Thiery *et al.*, 1973; Thiery *et al.*, 1973; Soave *et al.*, 1973); thymus (Laurila and Laskowski, 1957); tumor cells (Georgatsos, 1967; Ip and Sung, 1968); and brain (Rosen-

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