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INHIBITION OF HEXOKINASE BY MULTISUBSTRATE ANALOGS

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Summary

The compounds P^1-(adenosine-5′)-P^3-(glucose-6) triphosphate (Ap_3 glucose) and P^1-(adenosine-5′)-P^4-(glucose-6)tetraphosphate (Ap_4 glucose) were synthesized as possible transition-state analogs for hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1). Both compounds were inhibitors of this enzyme, competitive against ATP and apparently uncompetitive against glucose. The inhibition constants for Ap_3 glucose and Ap_4 glucose were 0.43 mM and 0.37 mM, respectively. These results indicate that the inhibitors do not appreciably bind to hexokinase until the glucose binding site is filled. The sugar portion of the inhibitors therefore does not contribute to binding, and the compounds are acting as ATP analogs. Ap_3 glucose and Ap_4 glucose are also slow substrates for glucose-6-phosphate dehydrogenase.

Current theory of enzymatic catalysis predicts that during the course of reaction, substrates in the transition state will be bound to the enzyme much more tightly than their individual binding capabilities [1]. Thus, stable compounds that closely resemble the transition state of a particular enzyme might be anticipated to exert profound inhibition. In fact, a number of analogs of transition states are known which display exceptionally low K_i values [2]. For two-substrate enzymes these compounds often consist of both substrate molecules joined together by a covalent bond at the reaction site. Such compounds, also known as multi-substrate analogs, provide unique tools for enzyme kinetics studies, because they are able to cover the binding sites of both substrates. There are as yet very few kinetic studies of enzymes using multisubstrate analogs.

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes a phosphoryl transfer from ATP to glucose to give ADP and glucose 6-phosphate. The mechanism of this enzyme has long been a subject of controversy [4—7], but the most recent evidence indicates a random mechanism with strong bind-

ing synergism between both substrates, ATP and glucose [4]. We have synthesized the candidate transition state analogs P^1-(adenosine-5′)-P^3-(glucose-6)-triphosphate (Ap_3 glucose) and P^1-(adenosine-5′)-P^4-(glucose-6)tetraphosphate (Ap_4 glucose). By studing the interaction of these inhibitors with hexokinase, we hoped to obtain information not only about the mechanism of substrate binding to this enzyme, but also about the kinetic behavior of multisubstrate analogs in an enzyme system with an unusually complex mechanism.

In this report, we describe the synthesis of these compounds and their interaction with hexokinase as measured by initial velocity enzyme kinetics.

Materials and Methods

Yeast hexokinase was purchased from Boehringer, as were lactate dehydrogenase, pyruvate kinase, phospho*enol*pyruvate and glucose 6-phosphate dehydrogenase. NADP, NADH, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and glucose were from Sigma, and ATP from P-L Biochemicals. 1,1′-Carbonyldiimidazole was obtained from Aldrich Chemical Company.

All reactions were followed at 340 nm using a Beckman DU monochrometer with a deuterium lamp, a Gilford optical density converter, and a 10 mV Leeds and Northrup recorder. Full scale sensitivity was 0.2 absorbance units with a chart speed of 3.0 to 6.0 inches per min. The cell compartment was maintained at 25.0°C by means of thermospacers. The reaction mixtures were made up to 2.9 ml in a 1.0 cm cuvette and allowed to equilibrate to 25°C in the cell compartment. The reaction was started by addition of 0.1 ml of hexokinase on an adder-mixer, and the initial velocity was determined as ΔA_{340} per min from the slope of the recorded line.

Each 3 ml cuvette contained 50 mM PIPES (pH 7.0), 20 units of pyruvate kinase, 55 units of lactate dehydrogenase, 167 mM NADH, 0.71 mM phospho*enol*pyruvate, 20 mM potassium acetate and 1.4 units of hexokinase. A concentration of 1.0 mM free magnesium acetate was maintained. Glucose and MgATP were the variable substrates.

Data processing. Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations and linear plots were obtained in all cases.

Data conforming to linear competitive inhibition and linear uncompetitive inhibition were fitted to Eqns. 1 and 2 respectively

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

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

K_{is} and K_{ii} are the apparent inhibition constants for slope and intercept. Experimental data were fitted to the respective equations by the least squares method, assuming equal variance for the velocities, with digital computer using the Fortran programs of Cleland [8]. The points in the double reciprocal plots are experimentally determined values, while the lines drawn through these points are calculated by the computer fit to the rate equations.

Synthesis of Ap_3 glucose. An extension of the method of Hoard and Ott [9] was used to synthesize the triphosphate. Glucose 6-phosphate and ADP were converted to their mono-*n*-pentylammonium salts by the following procedure. An aqueous solution of the compound was passed through a Dowex 50W-X4 column in the pyridinium form. To the eluate was added one equivalent of tri-*n*-pentylamine. The solution was the concentrated in vacuo to a syrup. Water was removed by repeated addition and re-evaporation of dry pyridine.

The tri-*n*-pentylammonium salt of ADP (1 mmol) was dissolved in 5 ml of dimethylformamide that had been previously dried over molecular sieves. 1,1′-Carbonyldiimidazole (80 mg; 5 mmol) was added, and the mixture stirred for 12 h in a stoppered flask. Methanol (0.33 ml; 8 mmol) was added to the solution to decompose excess reagent. After 30 min at room temperature, the mixture was treated with 2 mmol of glucose 6-phosphate (tri-*n*-pentylammonium salt) and stirring was continued for an additional 18 h. An equal volume of methanol was added, and after 30 min, the solvent was removed by evaporation at reduced pressure. The residue was placed on a DEAE-cellulose column (3 × 30 cm), previously equilibrated with triethylammonium bicarbonate. Elution was carried out using a linear gradient of 0—0.5 M triethylammonium bicarbonate, pH 7.5. The volume was 1 liter in each chamber, and 15 ml fractions were taken. The product was resolved into two major ultraviolet absorbing peaks with relative areas of about 70 : 30, with the larger peak eluting in fraction 25—75, and the smaller in fractions 125—250. The first peak was analyzed for phosphorus by the method of Fiske and SubbaRow [10], and for glucose by the method of Park and Johnson [11]. The resulting adenosine : phosphorus : glucose ratio was calculated to be 1.0 : 2.9 : 1.1 (theoretical ratios are 1 : 3 : 1). The second peak had lower base: phosphorus ratio (1 : 2.2) and was not fully identified. No glucose 6-phosphate could be detected on a paper chromatogram of the first product peak by a molybdate spray test for phosphates [12]. The product had an R_f value of 0.30 on Whatman no. 40 paper, using isopropanol/0.25 M ammonium bicarbonate, 65 : 35. ADP in the same system had an R_f of 0.45.

Ap_4 glucose. A method identical to that described above was used to synthesize this compound, except that ATP was substituted for ADP. R_F values were 0.20 for the product and 0.25 for ATP. The adenosine : phosphorus : glucose ratio was found to be 1.0 : 3.9 : 1.0 (theoretical 1 : 4 : 1).

Results

Ap_3 glucose was not a substrate for hexokinase, but was found to be an inhibitor, giving linear competitive kinetics with respect to MgATP. However, the degree of inhibition (Table I) was not of the order expected for a transition state analog that should have a K_i value substantially lower than the K_m values of the substrates; on the contrary, the observed K_i was quite similar to the K_m value of the substrate ATP. With glucose as the variable substrate, Ap_3 glucose gave an apparent uncompetitive inhibition pattern.

Recently, potent inhibition of the enzyme adenylate kinase was achieved with the compound Ap_5A [13]. The extra phosphate group in this transition state analog was thought to compensate for the increased bond length of pen-

TABLE I

INHIBITION OF HEXOKINASE BY THE MULTISUBSTRATE ANALOGS

Compound	Inhibition vs. ATP	Inhibition vs. Glucose	K_i * (mM)
Ap_3 glucose	Competitive	Uncompetitive	0.43
Ap_4 glucose	Competitive	Uncompetitive	0.37

* The K_m for ATP was 0.20 mM.

tavalent phosphorus in the transition state. To test this hypothesis on hexokinase, we synthesized Ap_4 glucose, which also contains an additional phosphate group between the two substrate moieties. However, this compound showed only moderate binding to hexokinase with a K_i close to that of Ap_3 glucose (Table I), and likewise was competitive with respect to MgATP and uncompetitive with respect to glucose.

During the course of a coupled enzyme assay for hexokinase involving the use of glucose-6-phosphate dehydrogenase [4], it became apparent that Ap_3 glucose and Ap_4 glucose are slow substrates for this enzyme. The maximum velocity was 5% of that using the normal substrate and K_m values were on the order of 1 mM, compared to 0.05 mM for glucose 6-phosphate. Thus, glucose-6-phosphate dehydrogenase is not specific for an unsubstituted phosphate group on the glucose. The rates measured for all hexokinase reactions using this assay were corrected for this background rate.

Discussion

The results described here illustrate that compounds incorporating the binding sites of both substrates, whether they are true transition state analogs or "multisubstrate analogs" [1], can be used to distinguish between possible binding mechanisms. If an enzyme had the simplest type of random mechanism with non-synergistic binding of substrates, compounds of this type would be able to bind to both sides of the free enzyme, and we would expect that (1) competitive inhibition against both substrates would be observed; (2) K_i values calculated from both inhibition patterns would be identical; and (3) since binding points in both sites are utilized, the binding of the inhibitor should be considerably tighter than that for each substrate alone. Such results were in fact obtained for the inhibition of phenylalanyl-tRNA synthetase by phenylalanyl-adenylate [3], an analog of the aminoacyladenylate transition state and a potent inhibitor of this enzyme. Phenylalanyl-tRNA synthetase had been shown by other kinetic methods to have a random mechanism of substrate binding [3]. Similarly, adenylate kinase is very strongly inhibited by Ap_5A ($K_i = 10^{-8}$), an inhibitor which is competitive against both substrates, AMP and MgATP [13], and thus binds to both sites. Based on these observations, we would predict that AMP and MgATP bind to adenylate kinase in a random and nonsynergistic manner.

The mechanism of hexokinase obviously is considerably more complex than of the enzymes cited above. The uncompetitive patterns obtained with glucose as the variable substrate indicate that at lower glucose concentrations, there is

only weak binding of the inhibitors, but the binding becomes progressively tighter with increasing glucose concentration. Thus, Ap_3 glucose and Ap_4 glucose are acting as ATP analogs which do not bind appreciably to the free enzyme, but show significant binding only when the glucose site is filled, which is consistent with recent findings [4] that certain sugars cause a 40-fold tightening of chromium-ATP binding, as well as a 40-fold decrease in the dissociation constant for MgATP in the ATPase reaction catalyzed by hexokinase [14]. The bimolecular dissociation constant of both Ap_3 glucose and Ap_4 glucose in the absence of glucose therefore should be on the order of 10 mM if they are indeed mimicking the substrate ATP. Inhibition patterns similar to those reported here were obtained by Hohnandel and Cooper [15] for 9-(β-D-glucopyranosyl) adenine 6′-triphosphate, an analog of ATP in which the ribose moiety has been replaced with a glucose. These observations indicate that the glucose moiety of the inhibitors does not contribute appreciably to binding. Because binding is the same regardless of the number of phosphate groups, it is likely the sugar portion simply juts out into the medium.

On the basis of the proposed random mechanism of substrate binding to hexokinase [4], one might have expected the inhibition by Ap_3 glucose and Ap_4 glucose to be non-competitive with respect to glucose. However, the kinetics are complicated by the aforementioned 40-fold binding synergism between the substrates. If the dissociation constant for the inhibitors were 40-fold lower in the presence of glucose, the slope effect in a noncompetitive pattern would be difficult to detect and thus would result in the apparent uncompetitive inhibition.

It was further demonstrated that both substrates, glucose and ATP, must be in the active site before hexokinase will undergo the conformational changes that lead to tightening of the binding [4]. Obviously, the glucose portion and the ATP portion of Ap_3 glucose and Ap_4 glucose are not able to act in concert to induce these conformational changes, otherwise the binding of these inhibitors would be more in line with that expected of a multisubstrate analog. Since the transition states of hexokinase and other phosphate-transferring enzymes are thought to have a linear trigonal bipyramid geometry [16], probably the tetrahedral bond angles in the phosphate bridges of these inhibitors do not allow simultaneous binding of the sugar portion and the nucleotide portion to the active site. In the case of adenylate kinase, placing an extra phosphate group between the substrate portions (Ap_5A) resulted in greatly increased binding compared to Ap_4A. In contrast, the extra phosphate group of Ap_4 glucose was not able to compensate for the unfavorable tetrahedral bond angles. This is consistent with the hypothesis that the glucose portion is not in contact with the enzyme when the inhibitors bind. It is also possible that a free 6-hydroxyl group on the glucose is required to initiate the required conformational changes.

Multisubstrate and transition state analogs have not heretofore been widely used in the study of enzyme kinetics. In this paper, we have shown that valuable supplementary data can be obtained through the use of such compounds. Furthermore, in cases where substrate analogs are not readily available, joining both substrate molecules together through a covalent bond may furnish an inhibitor which will allow considerable information to be obtained about the enzyme mechanism.

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