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INHIBITION OF BRAIN HEXOKINASE BY A MULTISUBSTRATE ANALOG RESULTS FROM BINDING TO A DISCRETE REGULATORY SITE

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SUMMARY: P^1-(adenosine-5')-P^3-(glucose-6)-triphosphate (Ap_3glucose) is a linear uncompetitive inhibitor vs glucose and a linear mixed inhibitor vs ATP of brain hexokinase, an inhibition pattern inconsistent with binding of Ap_3glucose to the catalytic site when either the rapid equilibrium random or ordered sequential mechanism, which have been proposed for this enzyme, is considered. It is concluded that inhibition results from binding to a discrete regulatory site. The apparent ability of the regulatory site to accomodate both hexose and nucleotide moieties is consistent with suggestions by previous investigators that the regulatory site on mammalian hexokinases may have evolved from what was originally a catalytic site.

It is well established that the hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) reaction proceeds via a ternary complex, i.e., both ATP and glucose must be simultaneously present on the enzyme for the reaction to occur (1). Thus a compound combining the structural features of both ATP and glucose might serve as an inhibitory multisubstrate analog (2,3) for hexokinase, and inhibition of yeast hexokinase by such compounds was previously reported (4). Because of the markedly different properties of yeast and mammalian hexokinases (1), we thought it useful to directly examine the effect of multisubstrate analogs on brain hexokinase, an enzyme which has been the object of continuing research interest in our laboratory. Accordingly, we have synthesized P^1-(adenosine-5')-P^3-(glucose-6)-triphosphate, hereafter referred to as Ap_3glucose, and examined it as an inhibitor of rat brain hexokinase (5). While this study was in progress, Hampton et al. (6) reported that Ap_3glucose (and the homolog, Ap_4glucose) was a rather poor inhibitor of mammalian hexokinases, including the Type I isozyme predominant in brain. In contrast, we have found Ap_3glucose to be a reasonably effective (apparent K_i < 1 mM) inhibitor of brain hexokinase and have characterized this

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inhibition vs both substrates. The results of this study are of interest with respect to defining the nature of a discrete regulatory site thought to exist on this enzyme.

MATERIALS AND METHODS

Rat brain hexokinase was purified according to Chou and Wilson (5). Activity was assayed at 23° by monitoring NADH oxidation spectrophotometrically in a 1 ml reaction mixture containing 40 mM Tris chloride (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 10 mM thioglycerol, 1 mM phosphoenol pyruvate, 0.11 mg NADH, 9 units beef heart lactate dehydrogenase, 2 units rabbit muscle pyruvate kinase, and concentrations of glucose, ATP, and Ap_3glucose as indicated in the figures. Primary kinetic data were analyzed by the weighted least squares method of Wilkinson (7) and secondary plots evaluated by a non-weighted least squares method using computer programs kindly made available by Dr. C.H. Suelter; lines drawn in the figures are as determined by these analyses.

Ap_3glucose was synthesized as previously described (4) using D-[1-^{14}C]-glucose-6-P (New England Nuclear). The compound migrated as a single radioactive species which coincided with a single UV-absorbing spot after electrophoresis on cellulose thin layer plates with a pyridine:acetic acid:H_2O (1:10:189), pH 3.5, buffer; it was confirmed that this product was readily distinguished from glucose-6-P and adenine nucleotide standards under these conditions. Adenine content was estimated based on absorbance at 259 nm using a molar extinction coefficient of 15.4×10^3 (8). Phosphate content was determined according to Hasegawa et al. (9), and glucose content from the known specific activity of the glucose-6-P used in the synthesis. The glucose:adenine:phosphate ratio was 1:0.93:3.2, which compares favorably with the 1:1:3 ratio expected for Ap_3glucose. These results indicate that the isolated compound was indeed Ap_3glucose, and free of detectable amounts of UV absorbing (e.g., adenine nucleotides) or radioactive (e.g., free glucose-6-P) contaminants that might influence the kinetic results.

RESULTS AND DISCUSSION

Ap_3glucose is a linear uncompetitive inhibitor vs glucose (Fig. 1), implying that Ap_3glucose can bind only to complexes which already include glucose, and a linear mixed inhibitor vs ATP (Fig. 2), implying that binding of Ap_3glucose and ATP are not mutually exclusive (in which case competitive inhibition would be expected).

If Ap_3glucose were acting as a multisubstrate analog binding at the catalytic site of brain hexokinase and the mechanism were rapid equilibrium random as proposed by Fromm and colleagues (1), the inhibition pattern would be predicted to be competitive vs both ATP and glucose; such inhibition has been observed with a multisubstrate analog of adenylate kinase, an enzyme believed to operate by a random addition mechanism (10,11). This clearly is not the case here. Alternatively, Ap_3glucose might bind only via a part of its structure (e.g. the "ATP" part or the "glucose" part) and hence act simply as a

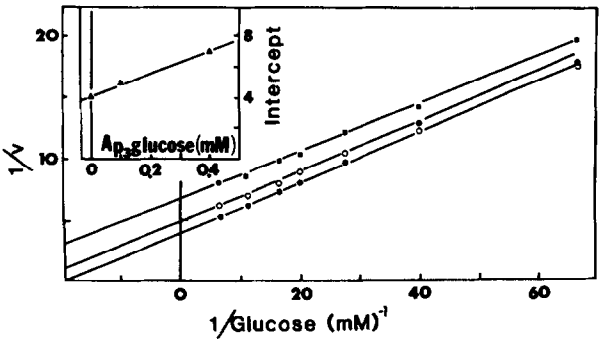


Fig. 1. Linear Uncompetitive Inhibition of Brain Hexokinase by Ap_3glucose. Assays were as described in the text and contained 4.4 mM ATP, the indicated concentration of glucose, and 0 (●), 0.10 (○), or 0.40 (■) mM Ap_3 glucose. Intercepts (from the 1/v axis) are plotted vs Ap_3 glucose concentration in the inset; extrapolation to the abscissa yields a value of 0.6 mM as the apparent K_i value.

substrate analog. In this case, Ap_3glucose would be predicted to be competitive vs one substrate and noncompetitive vs the other, again in contrast to the observed results. It has also been suggested that discrete (from the catalytic site) regulatory sites for binding of nucleotides (1) and glucose-6-P (12,13) exist on brain hexokinase. Thus Ap_3glucose might exert its inhibitory action by binding to one (or both) of these alternative sites. This would seem to be the only way to reconcile the observed results with a rapid equilibrium random addition mechanism, e.g.

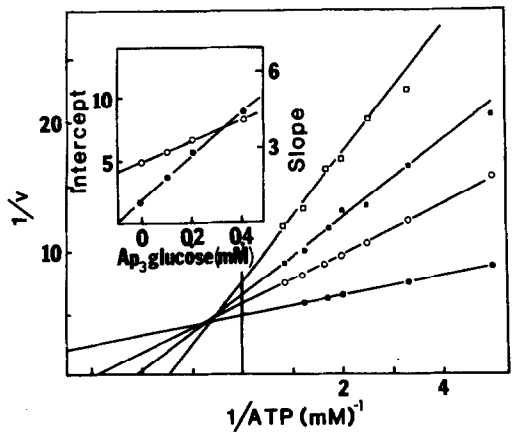
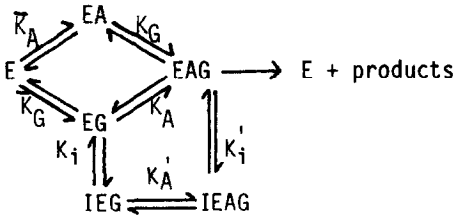


Fig. 2. Linear Mixed Inhibition of Brain Hexokinase by Ap_3glucose. Assays were as described in the text and contained 0.33 M glucose, the indicated concentration of ATP, and 0 (●), 0.10 (○), 0.21 (■), or 0.42 (□) mM Ap_3 glucose. Secondary plots of the slopes (●) and intercepts from the 1/v axis (○) vs Ap_3glucose concentration are shown in the inset; extrapolation of these plots to the abscissa gave values of 0.1 mM and 0.6 mM, respectively, for the apparent K_i values.



where E,A,G, and I represent enzyme, ATP, glucose, and Ap_3glucose, respectively, and the K values are dissociation constants for the relevant complexes. If all binding steps are at equilibrium, the rate equation is:

$$\frac{V_{max}}{v} = 1 + \frac{\bar{K}_G K_A}{(A)(G)} + \frac{K_G}{(G)} + \frac{K_A}{(A)} + \frac{K_A (I)}{K_i} + \frac{(I)}{K_i'}$$

and inhibition by Ap_3glucose is predicted to be uncompetitive vs glucose and mixed vs ATP, in accord with the observed results.

Gregoriou et al. (14) have recently proposed an ordered mechanism, glucose binding first, for Type II hexokinase of muscle, and suggested that an ordered mechanism is also applicable to the Type I isozyme, i.e., to brain hexokinase. Glucose and ATP can bind independently to hexokinase (15), which may seem inconsistent with an ordered kinetic mechanism, glucose adding first. However, an ordered mechanism which includes formation of an inactive enzyme-second substrate complex (hexokinase - ATP in the present case) can yield initial velocity kinetic patterns identical to those expected for a rapid-equilibrium random addition mechanism (16), though these two mechanisms could, in principle, be distinguished by alternative kinetic methods (17). At subsaturating levels of the leading substrate, an ordered mechanism with formation of an inactive enzyme-second substrate complex is predicted to lead to substrate inhibition as the concentration of the second substrate is increased (18). Based on the results of Purich et al. (18), substrate inhibition of brain hexokinase (5) would become evident only at subsaturating glucose and at ATP levels more than 10 X K_m (app), considerably greater than those used in most kinetic studies. However, the results of Copley and Fromm (Fig. 1 in ref. 19) do suggest the presence of substrate inhibition as ATP is

increased to very high levels at low glucose concentration, i.e., under the conditions expected to permit detection of this effect (18).

An ordered mechanism consistent with available results can be obtained simply by deleting the pathway directly connecting the enzyme-ATP complex with the ternary complex (corresponding to K_G) in the above scheme. The corresponding rate equation is indistinguishable in form from the equation for the rapid equilibrium random addition mechanism, and thus again leads to prediction of an inhibition pattern consistent with the present results. As with the random mechanism, the observed inhibition pattern is inconsistent with Ap_3glucose binding at the catalytic site. Thus, if Ap_3glucose could effectively mimic both glucose and ATP (i.e., bind to free enzyme to yield a pseudo ternary complex) or bind only to the glucose site *via* its hexose moiety, the predicted inhibition would be competitive *vs* glucose. Alternatively, if Ap_3glucose served only as an ATP analog, it would be competitive vs ATP. These predictions are at variance with the observed results.

Although the present study does not permit resolution of the controversy over the mechanism, it does provide compelling evidence for the existence of a discrete (from the catalytic site) regulatory site on brain hexokinase, for it is not possible to reconcile the inhibition pattern of Ap_3glucose with either an ordered or a random mechanism where Ap_3glucose is considered to bind at catalytic site regions normally accomodating glucose and/or ATP. Gregoriou *et al.* (14) proposed the existence of discrete regulatory sites for *both* ATP and glucose-6-P, but pointed out that the absence of marked substrate inhibition by ATP indicates that binding of ATP to the effector site is relatively weak. Binding of glucose-6-P, however, is quite avid, resulting in the potent inhibition which is competitive *vs* ATP and mixed *vs* glucose (1). We suggest that the regulatory ATP and glucose-6-P binding sites proposed by Gregoriou *et al.* (14) may be in close proximity (a feature discussed further below) and that Ap_3glucose can bind simultaneously to both sites resulting in an inhibition pattern that is somewhat different from that seen with glucose-6-P alone. These ligands are, however, similar in that their binding is facilitated by

prior binding of glucose (14,20,21, this paper) and that both ligands have a detrimental effect on binding of ATP at the catalytic site (1,15, this paper).

Several authors (14,22-26) have speculated that mammalian hexokinases, which consist of a single polypeptide chain with mol. wt. approx. 100,000 daltons and show potent inhibition by glucose-6-P, represent the evolutionary products of duplication and fusion of a gene for an ancestral hexokinase similar to mammalian glucokinase and yeast hexokinase, which consist of 50,000 dalton polypeptide chains and do not show potent inhibition by glucose-6-P. There is support for such a relationship to be found in peptide mapping studies (27) and comparison of amino acid compositions (25,26). It is suggested (14,22-26) that an allosteric regulatory site evolved from what was one of two catalytic sites in the original "fused" protein. The original catalytic site obviously could bind nucleotides as well as hexose moieties, and Gregoriou et al. (14) have noted that this may account for the existence of a residual ATP-binding (though of low affinity) regulatory site as well as the high affinity regulatory site for glucose-6-P. If these sites were indeed evolved from a precursor catalytic site, it may be expected that they would be in close proximity and hence the simultaneous binding of Ap_3glucose to both sites is a reasonable possibility. In this regard, it is interesting to note that Ap_3glucose has been reported to inhibit yeast hexokinase competitively vs ATP and uncompetitively vs glucose (4), a pattern not too dissimilar from that seen here with brain hexokinase. Could it be that one of the catalytic sites on yeast hexokinase (a dimer of 50,000 mol. wt. subunits) is already predisposed to influence the other catalytic site in a manner similar to the interactions occurring in brain hexokinase? Such a predisposition might have been honed by evolutionary processes to yield the sensitive regulation that characterizes mammalian hexokinases.

ACKNOWLEDGEMENT

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