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ON THE EXISTENCE OF A NUCLEOTIDE-BINDING REGULATORY SITE ON BRAIN HEXOKINASE

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SUMMARY: Binding of ADP to rat brain hexokinase provided protection against inactivation of the enzyme by glutaraldehyde or by chymotryptic digestion. Graphical analysis of the inactivation experiments was, in both cases, consistent with the existence of a single ADP binding site and a $K_d \approx 3mM$ for the hexokinase-ADP complex. Both Cibacron Blue F3GA and tetraiodofluorescein, previously found to have a general affinity for nucleotide binding sites, were competitive (vs. ATP) inhibitors of the enzyme, suggesting that they bound only to the site occupied by the nucleotide substrate, ATP. While alternate interpretations cannot be excluded, it is felt that these results are most consistent with the view that there is a single nucleotide binding site on the enzyme. They thereby may serve to stimulate a search for alternative explanations for the complex inhibitory pattern of ADP which had previously been attributed to the existence of two ADP binding sites on the enzyme (J. Ning, D.L. Purich, and H.J. Fromm, J. Biol. Chem. 244, 3840-3846 (1969).

One of the several unresolved questions concerning the kinetic mechanism and regulation of brain hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1.) deals with the observed inhibition pattern of the product ADP. Although based on the rapid equilibrium random mechanism proposed for the enzyme one would predict competitive inhibition vs. both ATP and glucose, the observed pattern is mixed inhibition (1,2). Different explanations for this have been advanced (1-3) but the matter is, to our knowledge, still unresolved.

Another observation that adds still further to the complexity is that the inhibition is not linearly dependent on ADP concentration, but rather a higher order dependence. The explanation put forth by Fromm and his colleagues (1) is based on the postulation of two binding sites for ADP, one being the

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catalytic site and the second being a distinct allosteric regulatory site which binds ADP (and other nucleotides) but not ATP. Although Purich and Fromm (2) have stated that this higher order dependence on [ADP] "cannot be rationalized in terms of a single binding site", Storer and Cornish-Bowden (4) have proposed a model for rat liver glucokinase in which such higher order dependence on [ADP] is accounted for without the postulation of a separate allosteric regulatory site.

An obvious approach to resolving the question as to the number of binding sites for ADP would be direct binding studies (e.g. equilibrium dialysis, or similar method). However, kinetic studies (1) suggest a dissociation constant (K_d) in the mM range for the hexokinase-ADP complex (a suggestion supported by the results presented here), and direct binding studies become technically difficult with ligands for which the enzyme has such low affinity. We have, therefore, chosen two alternative approaches: a) based on earlier work (5,6) in which protective effects against various inactivating agents were used to detect ligand-induced conformational changes and estimate K_d values for the hexokinase-ligand complexes, we have examined the effect of ADP on inactivation by glutaraldehyde and by chymotryptic digestion, the rationale being that multiple binding sites for ADP might introduce complexity which would be detectable in analysis of the results; b) we have determined the inhibition pattern seen with Cibacron Blue F3GA and tetraiodofluorescein, two ligands which have been shown (7-9, and references therein) to have a general affinity for nucleotide binding sites; hence, if there were indeed two such sites on brain hexokinase, one might also predict mixed inhibition analogous to that seen with ADP.

MATERIALS AND METHODS

Rat brain hexokinase was prepared and assayed as described previously (10). Glutaraldehyde (50% solution) was purchased

from Electron Microscopy Sciences (Ft. Washington, PA), chymotrypsin from Worthington Biochemical Corp. (Freehold, NJ) and other biochemicals from Sigma Chemical Co. (St. Louis, MO). The procedures used in the chymotryptic digestion (5) and glutaraldehyde inactivation (6) were as given in the indicated references. Evaluation of rate constants and dissociation constants were as given earlier (6).

RESULTS

Protection by ADP against Chymotryptic Digestion and Glutaraldehyde Inactivation--As in previous studies (5,6) inactivation by chymotrypsin followed apparent first order kinetics. ADP was found to exert a marked protective effect against chymotryptic digestion, and from the concentration dependence of the protective effect, a K_d can be estimated (6). The results of a typical experiment are plotted in Fig. 1 and indicate a K_d of 1.9 mM for the hexokinase - ADP complex. From four such experiments, the average $K_d \pm$ S.D. was 3.5 ± 1.4 mM.

ADP also offered marked protection against inactivation by glutaraldehyde. From the results of the experiment plotted in Fig. 1, a K_d of 3.6 mM was estimated. From five determinations, the average $K_d \pm$ S.D. was 3.2 ± 0.9 mM.

Inhibition by Cibacron Blue F3GA and Tetraiodofluorescein--It has previously been reported (8) that Cibacron Blue F3GA was a competitive (vs. ATP) inhibitor of rat brain hexokinase, with an estimated $K_i \simeq 30$ μM. The present study demonstrated that tetraiodofluorescein was also a competitive inhibitor of brain hexokinase (Fig.2), with a $K_i \simeq 26$ μM.

DISCUSSION

Based on the rationales stated at the beginning of this paper, these results do not support the view that there are two distinct binding sites for ADP on the brain hexokinase molecule. If there

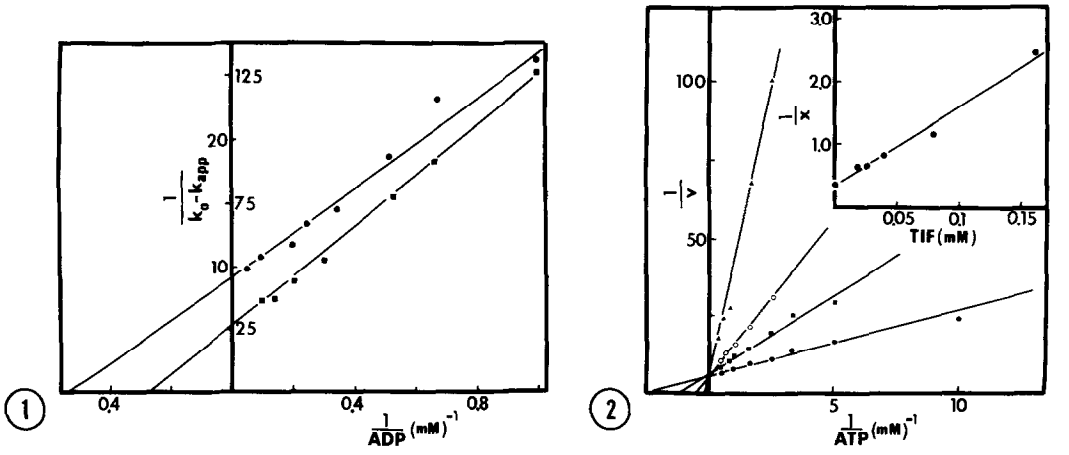

Figure 1. Dissociation Constant for the Hexokinase-ADP Complex Determined by the Glutaraldehyde Inactivation and Chymotryptic Digestion Methods. The procedures were as described previously (5,6); k_o is the rate constant for inactivation in the absence of added ADP, while k_{app} is the rate constant observed in the presence of the indicated concentration of ADP. The "best fit" lines were determined by least squares analysis; extrapolation to $\frac{1}{k_o - k_{app}} = 0$ yields [ADP] = K_d. Glutaraldehyde inactivation (■), numbers to right of ordinate, $k_o = 0.047\ min^{-1}$; chymotryptic digestion (●), numbers to left of ordinate, $k_o = 0.109\ min^{-1}$.

Figure 2. Inhibition of Rat Brain Hexokinase by Tetraiodofluorescein. [Tetraiodofluorescein] : 0, (●); 40μM, (■); 80μM, (○); 160μM, (▲). The inset is a replot of the reciprocals of the intercepts on the 1/ATP axis vs. [tetraiodofluorescein] (TIF); extrapolation to the abscissa yields a value of 26μM as the K_i.

were different sites, they might reasonably be expected to differ in K_d values and in the relative amount of protection provided against glutaraldehyde and chymotrypsin. Such differences should have introduced nonlinearity into the plots and/or resulted in lack of agreement in K_d values determined by these different methods. However, analysis of the inactivation experiments failed to disclose any noticeable departures from the linear relationship expected if the protective effect of ADP resulted from a single binding function with a $K_d \simeq 3$mM. Furthermore, the agreement between the K_d values determined by two different methods (i.e. protection against glutaraldehyde and chymotrypsin) would be consistent with the

view that binding of ADP to this site conferred protection against both inactivating agents.

Similarly, if there were two binding sites for ADP, one might have expected both sites to also bind either (or both) Cibacron Blue F3GA or tetraiodofluorescein, previously shown to have an affinity for nucleotide binding sites in a wide variety of enzymes (7-9, and references therein). One would not predict the observed competitive inhibition pattern under such circumstances.

It is certainly obvious that the present results do not constitute proof that there is but a single ADP binding site, nor exclude the possibility that there are two such sites. But on the other hand, neither do the kinetic arguments (1,2) constitute proof for the existence of two sites, and alternate explanations are likely to be found (e.g.,4). The present report may at least provide a stimulus to the search for such alternate explanations.

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