Effect of Inorganic Phosphate on the Reverse Reaction of Bovine Brain Hexokinase†

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ABSTRACT: Kinetic studies were used to investigate the mode of brain hexokinase (EC 2.7.1.1, ATP:D-hexose 6-phosphotransferase) regulation by glucose 6-phosphate (glucose-6-P), ADP, and inorganic phosphate (P_i). A model for regulation of brain hexokinase by glucose-6-P and P_i had been proposed from initial-rate studies and binding experiments [Ellison, W. R., Lueck, J. D., & Fromm, H. J. (1975) *J. Biol. Chem. 250*, 1864–1871]. The results of the present investigation demonstrate that P_i is an activator of the brain hexokinase reaction when the reaction is studied in the nonphysiological direction. Evidence is presented which indicates that the back-reaction substrates and P_i can bind the enzyme simultaneously, and the suggestion is made that P_i binds to an allosteric site on the enzyme. These findings are in marked contrast to results obtained in the absence of ADP which convincingly demonstrate that glucose-6-P and P_i are mutually exclusive binding ligands for brain hexokinase. The kinetic data can be reconciled with the model for hexokinase regulation within the context of the well-established kinetic mechanism for brain hexokinase.

Hexokinase I, brain hexokinase, is accepted today as a pacemaker of glycolysis in brain tissue (Lowry & Passonneau, 1964) and in the erythrocyte (Rapoport, 1968). The observation of Tiedemann & Born (1959) that inorganic phosphate (P_i) increases the activity of glucose 6-phosphate (glucose-6-P) inhibited brain hexokinase provides an attractive model for coordinated glycolytic regulation in tissues that do not rely heavily on glycogen metabolism. That increased glycolytic rates can occur without large increases in glucose-6-P levels (Uyeda & Racker, 1965) may be explained on the basis of P_i activation of both glucose-6-P-inhibited hexokinase and phosphofructokinase (Passonneau and Lowry, 1962).

The suggestion has been made that brain hexokinase may be controlled by glucose and the entire nucleotide pool, in addition to glucose-6-P and P_i (Ning et al., 1969; Purich & Fromm, 1971). That the level of hexokinase in the cerebral cortical tissue far exceeds the amount required to meet the requirements of glycolysis is well established (McIlwain, 1966). Under normal physiological conditions, hexokinase I is markedly inhibited, 95–97%, and yet is capable of accelerated rates of activity for brief periods. Purich & Fromm (1971) found that glucose-6-P-inhibited hexokinase was dramatically deinhibited by P_i in the latter's physiological range, whereas this effect was diminished by nucleotides other than ATP. The suggestion was made that nucleotides, other than ATP, serve to moderate extreme increases in the glycolytic flux that might otherwise occur when the intracellular concentration of P_i increases.

One of the questions of major interest to us concerned the mode, at the molecular level, of glucose-6-P inhibition of brain hexokinase and its reversal by P_i within the context of the enzyme's kinetic mechanism. Hexokinase I exhibits rapid-equilibrium random Bi-Bi kinetics (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974) and is a monomer of molecular weight 97 000 (Easterby & O'Brien, 1973). The uninhibited or ADP-inhibited enzyme is insensitive to P_i when studied in the forward direction (Uyeda & Racker, 1965; Purich & Fromm, 1971; Kosow et al., 1973), and kinetic experiments have demonstrated that glucose-6-P is a linear competitive inhibitor of ATP (Ellison et al., 1975; Fromm & Zewe, 1962; Grossbard & Schimke, 1966) even at 100 times its inhibition constant. Ligand-binding experiments showed that P_i binds relatively tightly to hexokinase in the absence of glucose-6-P but that these two compounds exhibit mutually exclusive binding (Ellison et al., 1975). These and other binding studies suggest that only one molecule of glucose, glucose-6-P, and P_i is capable of binding per molecule of enzyme (Chou & Wilson, 1974; Ellison et al., 1974, 1975). A model, based upon the kinetic and binding studies, was presented in an attempt to explain the inhibitory effect of glucose-6-P on brain hexokinase and its reversal by P_i (Ellison et al., 1974). This model implies that hexokinase may exist in equilibrium either as the free enzyme or as the P_i-associated enzyme. The kinetic parameters of the two enzyme forms are similar, except in their ability to bind glucose-6-P, and it was suggested that the dissociation constant for glucose-6-P is relatively very high for hexokinase I to which P_i is bound. P_i appears to bind at an allosteric site on the enzyme, whereas glucose-6-P binds either at the active site or at a site that overlaps the catalytic site.

The present investigation was undertaken in an attempt to understand why glucose-6-P-inhibited hexokinase is highly responsive to P_i, whereas the inhibited enzyme in the presence of nucleotides, including ADP, is not (Purich & Fromm, 1971). This inability of the glucose-6-P-inhibited enzyme to respond to P_i in the presence of ADP may have important physiological implications.

Experimental Procedures

Materials. Glucose oxidase and horseradish peroxidase were purchased from Boehringer-Mannheim. Mutarotase was from Sigma, as was ADP, glucose-6-P, 4-aminoantipyrine, and Pipes buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonate)]. Distilled, deionized water was used in the preparation of all reagents. All other chemicals were of the highest purity available commercially.

Methods. Bovine brain hexokinase was purified by the method of Redkar & Kenkare (1972) and had a specific ac-

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tivity of 62 units/mg. A unit of activity is defined as 1 μmol of glucose-6-P produced per min at 28 °C in a total reaction volume of 3 mL under standard assay conditions. The standard assay contained 10 mM glucose, 5 mM ATP, 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8), 0.33 mM NADP, 7 mM $MgCl_2$, and 1.7 units of glucose-6-phosphate dehydrogenase in a total volume of 3 mL.

The purified enzyme was stored as previously described (Redkar & Kenkare, 1972) and was desalted before each experiment on a Bio-Gel P2 column, which was equilibrated with 100 mM Pipes, pH 6.0. The enzyme was found to be stable for at least 12 h after desalting. For initial-rate studies, the enzyme was diluted into 1 mg/mL bovine serum albumin in 100 mM Pipes, pH 6.0.

Aluminum, contaminating the commercial preparations of ADP and ATP, was removed by the procedure of Solheim & Fromm (1980) with 8-hydroxyquinoline in chloroform. P_i was removed from the nucleotides by passage of the solutions through a 150 × 2 cm Sephadex G-10 column.

The coupling enzymes were dialyzed against 5 mM NaCl in 10 mM Pipes buffer, pH 6.0, overnight before use to remove ammonium sulfate, which activated the reverse reaction.

Reaction rates in the nonphysiological direction were determined in a Cary 118c spectrophotometer by measuring the conversion of phenol and 4-aminopyrine to quinoneimine (Gierow & Jergil, 1980). For verification that the rates measured were not limited by the coupling enzymes, various concentrations of hexokinase were added to assays containing constant amounts of mutarotase, glucose oxidase, and peroxidase. Hexokinase concentrations used in the initial-rate experiments were from well within the linear portion of the velocity vs. enzyme profile. A typical assay contained 30 units of glucose oxidase, 10 units of peroxidase, and 20 units of mutarotase. The reactions were initiated by addition of brain hexokinase. Two controls were run to verify that changes in absorbance were due only to the reverse reaction catalyzed by hexokinase. One contained the total assay mixture without hexokinase, and the other contained hexokinase but no ADP or glucose-6-P. In each case, no change in absorbance was observed.

Initial-rate data were analyzed and fit to specific models by the weighted least-squares method, assuming equal variance of velocities, by using a computer program written in the OMNITAB language (Siano et al., 1975). The value of α was set equal to zero.

Results

We recently reported that the kinetics of the hexokinase I reaction from the nonphysiological direction were sequential Solheim & Fromm, 1981). A series of initial-rate experiments of the reverse reaction were undertaken to gain additional insight into the mechanism of regulation of hexokinase by glucose-6-P and P_i. Preliminary studies in which substrate concentrations were held constant at, or slightly above, their Michaelis constant values indicated that P_i activated brain hexokinase. This effect was readily discernible at 0.5 mM P_i, and activation increased until maximum velocity was reached between 10 and 15 mM P_i, where the P_i binding site seems to be saturated. Initial-rate studies, performed in the presence and absence of P_i, made it clear that the kinetic mechanism of the back-reaction of hexokinase I was sequential and that the kinetics were Michaelian. At glucose-6-P levels above 40 μM, substrate inhibition was in evidence. The results obtained in the absence of glucose-6-P inhibition (below 40 μM) resemble, quantitatively, the data obtained in the absence of P_i [Figures 1 and 2 in Solheim & Fromm (1981)].

Table I: Comparison of the Kinetic Parameters for the Reverse Reaction of Hexokinase I in the Presence and Absence of Inorganic Phosphate

parameter	no phosphate	2 mM phosphate
$K_{i,G6P}$ (μM)	13.1 ± 5.00	20.3 ± 4.10
K_{G6P} (μM)	8.5 ± 5.10	11.7 ± 3.40
$K_{i,ADP}$ (mM)	1.25 ± 0.530	0.928 ± 0.280
K_{ADP} (mM)	0.81 ± 0.180	0.570 ± 0.074
K_{EPL*} (mM)		2.00 ± 0.54[a]
K_{EPL} (mM)		0.49 ± 0.06[a]

[a] Calculated from the data of Figure 4 by using a value of 1.3 for α.

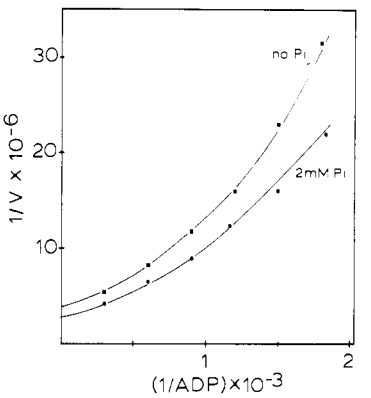


FIGURE 1: Plot of the reciprocal of the initial velocity (V, molar per minute) with respect to the reciprocal of the molar concentration of $MgADP^-$ in the absence (□) or presence (○) of 2 mM inorganic phosphate. $MgADP^-$ concentration was varied over the range of 0.56–3.33 mM. Free Mg^{2+} concentration was maintained at 10 mM. Glucose-6-P concentration was varied, in a constant ratio to $MgADP^-$ concentration, from 6.67 to 40 μM. Assays were performed at pH 6.0 (100 mM Pipes buffer) with 0.006 unit/mL enzyme.

Table I lists the kinetic parameters derived from the initial-rate data obtained in the presence and absence of P_i. The data described in Table I indicate that the changes in the kinetic parameters caused by P_i are small. These findings are of interest because, based upon the well-established ability of P_i to neutralize the inhibitory effect of glucose-6-P, one would have predicted that P_i should inhibit the reverse reaction. The primary effect of P_i on the back-reaction is on the Michaelis and dissociation constants for ADP and glucose-6-P with a small (less than 50%) increase in the maximal velocity. On the basis of the results depicted in Table I, P_i appears to weaken the binding of glucose-6-P, whereas this ligand enhances the binding of ADP. Although these effects are small and may not be significant, they contrast markedly with binding studies in the absence of ADP and with kinetic studies of the forward reaction in which ADP is omitted (Ellison et al., 1975).

To test the effect of P_i on the maximal velocity with a single experiment, we varied the substrate concentrations in a constant ratio in the presence and absence of P_i. The data obtained from these experiments, shown in Figure 1, were fit to eq 1. The computer-calculated maximal velocities varied

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_a}{[A]} + \frac{K_b}{c[A]} + \frac{K_{ia}K_b}{c[A]^2}\right] \quad (1)$$

between experiments but indicated that P_i activates the back-reaction of hexokinase by a factor of 1.2–1.4. In eq 1, v, V_1, K_a, K_b, K_{ia}, [A], [B], and c represent initial velocity, maximum velocity, Michaelis constant for A, Michaelis constant for B, dissociation constant for A, substrate A concen-

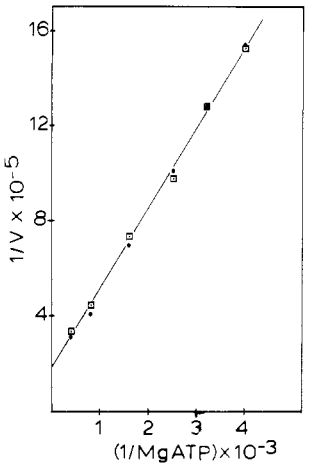


FIGURE 2: Plot of the reciprocal of the initial velocity (V, molar per minute) with respect to the reciprocal of the molar concentration of $MgATP^{2-}$ in the absence (○) or presence (□) of 2 mM inorganic phosphate. Free Mg^{2+} concentration was maintained at 2.5 mM. $MgATP^{2-}$ concentration was varied over the range 0.25–2.5 mM. Glucose concentration was varied, in a constant ratio to $MgATP^{2-}$ concentration, from 0.02 to 0.2 mM. Assays were performed in 100 mM Pipes buffer, pH 6, and included 0.005 unit of enzyme/assay.

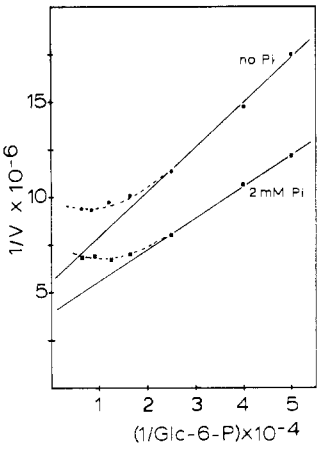


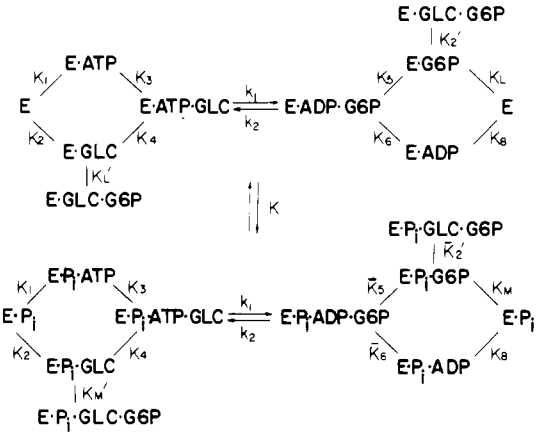
FIGURE 3: Plot of the reciprocal of the initial velocity (V, molar per minute) with respect to the reciprocal molar concentration of free glucose-6-P in the absence (○) or presence (□) of 2 mM inorganic phosphate. Glucose-6-P concentration was varied over the range 20–160 μM. $MgADP^-$ concentration was held constant at 1 mM, and free Mg^{2+} concentration was at 10 mM. Reactions were performed at pH 6.0 in 100 mM Pipes buffer.

tration, substrate B concentration, and substrate B to substrate A concentration ratio, respectively.

Because the effect of P_i on the forward reaction of brain hexokinase was evaluated at basic pH [i.e., pH 7.6 (Ellison et al., 1975)], a similar experiment was performed, varying glucose and ATP concentrations at a constant ratio, to verify that the P_i activation was not due to the change in pH. It can be seen in Figure 2 that P_i has no effect on the velocity or kinetic parameters of the uninhibited enzyme at this pH. The linear relationship shown in Figure 2 is what is to be expected for a Ping-Pong mechanism; i.e., the last term in eq 1 is eliminated. This finding is in harmony with the results reported at pH 7.6 by Fromm & Zewe (1962); however, the reaction mechanism is recognized to be sequential (Ning et al., 1969).

Although P_i activates the back-reaction of hexokinase to some extent, it has no effect on the excess substrate inhibition seen at levels of glucose-6-P above 40 μM (Figure 3). This finding suggests that there is a low-affinity glucose-6-P site associated with hexokinase that is insensitive to P_i activation.

Scheme I



The effect of P_i on ADP and glucose-6-P is illustrated in Figures 4 and 5. It can be seen from the insets to the figures that P_i acts as a hyperbolic activator with respect to both substrates. The major effect of P_i appears to be on the apparent maximal velocity in Figure 4 (intercept effect), whereas in Figure 5, the primary effect of P_i seems to be on the slopes of the double-reciprocal plots.

Discussion

Although the important role played by glucose-6-P, P_i, and nucleotides in the regulation of brain hexokinase has been recognized for many years, it was not until relatively recently that an attempt was made to quantitate the molecular mode of hexokinase regulation (Ellison et al., 1974, 1975). These investigations led to the model for hexokinase control presented in Scheme I from kinetic studies of the forward reaction of hexokinase and from binding studies. In the mechanism described by Scheme I, hexokinase exists as either the free enzyme or the P_i-associated enzyme. Because of the general agreement that the kinetic mechanism of brain hexokinase is rapid-equilibrium random Bi-Bi (Bachelard et al., 1971; Gerber et al., 1974; Purich & Fromm, 1971), it was suggested that the interconversion of the central ternary and quaternary complexes is rate limiting, the other steps being in rapid equilibrium. Because P_i did not seem to affect the kinetic parameters of the two enzyme forms in the absence of glucose-6-P, it was assumed that the rate-limiting step, k_1, is the same for the free and P_i-associated forms of the enzyme. Furthermore, on the basis of this same observation, the authors suggested that K, the dissociation constant for the E·P_i complex, is the same for P_i dissociation from all E–P_i–substrate and E–P_i–product complexes, except those of E·P_i·G6P and E·P_i·ADP·G6P where K_{EPL} applies and E·P_i·Glc·G6P is governed by K'_{EPL}. Because there is preferential binding of glucose-6-P to enzyme conformations devoid of P_i, the following relationships held: $K_M \gg K_L$ and $K'_M \gg K'_L$. The identities also held that $K_L \cdot K_{EPL} = K_M \cdot K$ and $K'_L \cdot K'_{EPL} = K'_M \cdot K$ and, thus, $K \ll K'_{EPL}, K_{EPL}$.

The kinetic equation for glucose-6-P inhibition of brain hexokinase and its reversal by P_i, based upon Scheme I, is

$$\frac{1}{v} = \frac{1}{V_1}\left\{1 + \frac{K_a}{[A]} + \frac{K_b}{[B]}\left(1 + \frac{Q}{K'_L \cdot f'(P_i)}\right) + \frac{K_{ia}K_b}{[A][B]}\left(1 + \frac{Q}{K_L \cdot f(P_i)}\right)\right\} \quad (2)$$

where V_1, K_a, [A], K_b, [B], [Q], and K_{ia} represent the maximal velocity, $K_{glucose}$, glucose concentration, K_{ATP}, ATP concen-

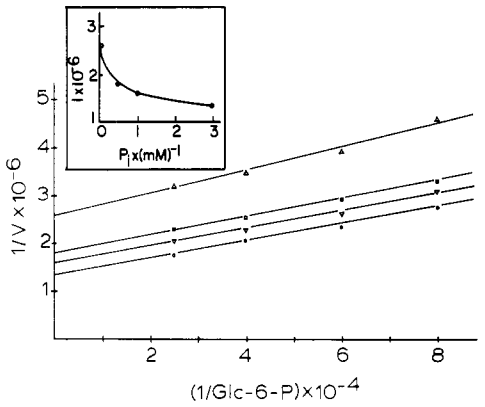


FIGURE 4: Plot of the reciprocal of the initial velocity (V, molar per minute) with respect to the reciprocal molar concentration of glucose-6-P in the absence (△) and presence of 0.5 (□), 1.0 (▽), and 3.0 mM (○) inorganic phosphate. Ionic strength was held constant by the addition of supplemental KCl. Glucose-6-P concentration was varied from 12.5 to 40 μM. $MgADP^-$ concentration was held constant at 0.75 mM, and free Mg^{2+} concentration was maintained at 10 mM. Assays were performed in 50 mM Pipes buffer, pH 6, with 0.04 unit/mL enzyme. Inset: A plot of intercepts (I) as a function of P_i concentration.

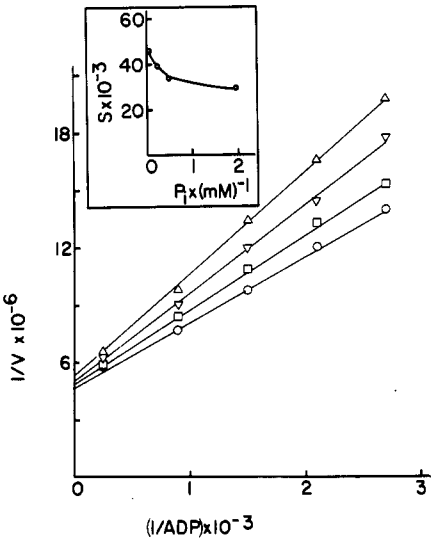


FIGURE 5: Plot of the reciprocal of the initial velocity (V, molar per minute) with respect to the reciprocal molar concentration of $MgADP^-$ in the absence (△) and presence of 0.25 (▽), 0.5 (□), and 2 mM (○) inorganic phosphate. Ionic strength was held constant by the addition of supplemental KCl. $MgADP^-$ concentration was varied from 0.37 to 3.33 mM. Free Mg^{2+} concentration was maintained at 10 mM. Assays were performed at pH 6 in the presence of 20 μM glucose-6-P. Inset: A plot of slopes (S) as a function of P_i concentration.

tration, glucose-6-P concentration and $K_{i,glucose}$, respectively. In addition, $f'(P_i)$ and $f(P_i)$ are defined as

$$f'(P_i) = \frac{K + [P_i]}{K + K'_L(P_i)/K'_M}$$

and

$$f(P_i) = \frac{K + [P_i]}{K + K_L(P_i)/K_M}$$

The mechanism outlined by Scheme I accounted for a number of observations made with hexokinase I by Ellison et al. (1974, 1975). These include inhibition by glucose-6-P and its reversal by P_i, lack of P_i inhibition or activation of the forward reaction in the absence of glucose-6-P, competitive inhibition of glucose-6-P relative to $MgATP^{2-}$, noncompetitive inhibition of the sugar phosphate with respect to glucose, and enhanced binding of glucose-6-P in the presence of glucose (but, binding only 1 mol of glucose-6-P per mol of enzyme). One observation that was not satisfactorily explained by this mechanism, but that is in agreement with the present study on the P_i regulation of hexokinase, is the ability of P_i to facilitate the approach to isotopic equilibrium by 55%. Since P_i was proposed to inhibit the binding of glucose-6-P to the enzyme, this result seemed incongruous. In light of the present data indicating the P_i-mediated activation of the reverse reaction, such a result would be expected.

The results obtained in the present study are consistent with the model shown in Scheme I with some modification of the mechanism. These modifications arise from the effect of ADP upon the enzyme and because the mechanism described by Scheme I was obtained in studies on the forward reaction, in the absence of ADP (Ellison et al., 1975). Because P_i and glucose-6-P are nearly mutually exclusive binding ligands (Ellison et al., 1974, 1975), one might expect that P_i would inhibit the back-reaction of hexokinase I. The data in Figures 1–5 indicate that just the opposite is true. The unpredicted effect of P_i on the kinetics of the reverse reaction undoubtedly is a consequence of ADP interaction with hexokinase. The effect of P_i in activating the back-reaction is in fact consistent with its well-established effects in the forward reaction.

Results of the present investigation are in harmony with the model shown in Scheme I, with only a few changes in the assumptions made regarding the relationships between certain dissociation constants.

The rate equation for the reverse hexokinase I reaction, with respect to Scheme I, is

$$\frac{1}{v} = \frac{1}{V_1}\left\{\frac{1 + [P_i]/K_{EPL}}{1 + \alpha[P_i]/K_{EPL}} + \frac{K_q(1 + [P_i]/K')}{[Q](1 + \alpha[P_i]/K_{EPL})} + \frac{K_p}{[P]}\left(\frac{1 + [P_i]/K_{EPL\bullet}}{1 + \alpha[P_i]/K_{EPL}}\right) + \frac{K_{iq}K_p}{[P][Q]}\left(\frac{1 + [P_i]/K}{1 + \alpha[P_i]/K_{EPL}}\right)\right\} \quad (3)$$

where K_q, $[Q]$, K_p, $[P]$, and K_{iq} represent the Michaelis constant for glucose-6-P, glucose-6-P concentration, the Michaelis constant for ADP, ADP concentration, and the dissociation constant for the E·G6P complex, respectively. K_{EPL} is the dissociation constant of P_i from the quaternary complex E·P_i·ADP·G6P, while K', $K_{EPL\bullet}$, and K represent the dissociation constants of P_i from the E·P_i·ADP complex, the E·P_i·G6P complex, and the E·P_i complex, respectively. α represents the fractional increase of the limiting value of V_{max} in the presence of saturating concentrations of P_i over the value of V_{max} in the absence of P_i. The following relationships can thus be shown to hold: $K_L K_{EPL\bullet} = K_M K$ and $K_{EPL\bullet} K_5 = K_5 K_{EPL}$.

The activation of the reaction in the presence of P_i comes about when several criteria regarding the dissociation constants are met. At a minimum, the following relationships must be valid:

$$\alpha > 1 \quad (4)$$

$$\frac{K_q}{[Q]}\left(\frac{K_{EPL}}{\alpha K'}\right) + \frac{K_{iq}K_p}{[P][Q]}\left(\frac{K_{EPL}}{\alpha K}\right) < \frac{K_q}{[Q]} + \frac{K_{iq}K_P}{[P][Q]} \quad (5)$$

$$\frac{K_p}{[P]}\left(\frac{K_{EPL}}{\alpha K_{EPL\bullet}}\right) + \frac{K_{iq}K_p}{[P][Q]}\left(\frac{K_{EPL}}{\alpha K}\right) < \frac{K_p}{[P]} + \frac{K_{iq}K_p}{[P][Q]} \quad (6)$$

These relationships arise from evaluation of eq 3 at infinite

P_i concentration by using either algebra or L'Hopital's rules for evaluating indeterminant expressions, and they assure decreases in the slope and intercept in double-reciprocal plots with increasing P_i concentration as seen in Figures 4 and 5.

The constant α value alone could cause the necessary difference between the two kinetic expressions since it appears in each term of the equation. Evaluating the term α has led to some difficulty. Several determinations as shown in Figure 1 have been attempted, and although each experiment yielded a value for α greater than 1, the significance of the difference, as evaluated by Student's t test, is questionable in at least some of the determinations. If α is assumed to be unity, a simpler kinetic expression results, as shown in eq 7:

$$\frac{1}{v} = \frac{1}{V_i}\left\{1 + \frac{K_q}{[Q]}\left(\frac{1 + [P_i]/K'}{1 + [P_i]/K_{EPL}}\right) + \frac{K_p}{[P]}\left(\frac{1 + [P_i]/K_{EPL^*}}{1 + [P_i]/K_{EPL}}\right) + \frac{K_{iq}K_p}{[P][Q]}\left(\frac{1 + [P_i]/K}{1 + [P_i]/K_{EPL}}\right)\right\} \quad (7)$$

where the kinetic parameters are defined as in eq 3. When this expression is evaluated at infinite P_i concentration, several simple relationships are defined that must be true to obtain P_i activation. These relationships are $K_{EPL} < K'$, $K_{EPL} < K_{EPL^*}$, and $K_{EPL} < K$.

It is clear from the results illustrated in Figures 4 and 5 that Figure 4 may be described as uncompetitive hyperbolic activation, whereas Figure 5 can be considered to be competitive hyperbolic activation. There is no statistically significant difference between the uncompetitive and noncompetitive models described by the data of Figure 4. Similarly, there is no significant difference between the noncompetitive hyperbolic activation model illustrated in Figure 5 and the analogous competitive case. Rationalization of the findings of Figures 4 and 5, within the context of the model shown in Scheme I, can be achieved by making either one of two different sets of assumptions regarding the dissociation constants described by eq 3 and 7. If it is assumed that Figure 4 is truly uncompetitive, $K_{EPL} = \alpha K'$ and $K_{EPL} = \alpha K$, and thus $K' = K$. If Figure 5 is truly competitive, the additional assumption, namely that $\alpha = 1$, is required. If $\alpha = 1$, and $K_{EPL} > \alpha K'$ and $K_{EPL} < \alpha K$ or $K_{EPL} < \alpha K'$ and $K_{EPL} > \alpha K$, the hyperbolic activation will be uncompetitive in Figure 4 and competitive in Figure 5, provided that the increasing function is exactly counterbalanced by the decreasing function. The data of Figure 1 suggest that $\alpha \neq 1$, and it is likely that P_i is in fact a noncompetitive hyperbolic activator with respect to ADP (Figure 5).

It follows from this discussion that the primary factor causing the hyperbolic activation observed in Figures 4 and 5 is the $(1 + [P_i]/K_{EPL^*})/(1 + \alpha[P_i]/K_{EPL})$ term in eq 3.

From the foregoing analysis, it may be concluded that the only difference between the model for brain hexokinase regulation proposed by Ellison et al. (1975) and that presented in the present study is the relationship $\alpha K_{EPL^*} > K_{EPL}$. Table I provides estimates of K_{EPL^*} and K_{EPL}, assuming $\alpha = 1.3$.

These assumptions are somewhat different from those proposed by Ellison et al. (1975), but only insofar as these earlier investigators did not include ADP in any of their experiments. The extension of this model to include the effect of P_i concentration on the reverse hexokinase I reaction provides a comprehensive model of regulation of brain hexokinase by sugar phosphate and P_i.

Purich et al. (1973) reported that brain hexokinase in the presence of substrates, glucose-6-P, ADP, and other nucleotides exhibits only a slight response to P_i in its physiological concentration range. They suggest that ADP and other intracellular nucleotides serve to dampen the effect of P_i on glucose-6-P-inhibited hexokinase I so as to moderate the flux through the hexokinase reaction. The results of this report are clearly in accord with this suggestion and stress the physiological importance of ADP in the regulation of the hexokinase reaction.

It had been assumed by Ellison et al. (1975) that an allosteric site for P_i exists on brain hexokinase. Tight binding of the ligand was demonstrated; however, it was also shown that P_i and glucose-6-P are mutually exclusive binding ligands. Data from the present report show that P_i is capable of activating, i.e., binding to the enzyme, in the presence of ADP and glucose-6-P. This observation supports the view that these three ligands can bind to brain hexokinase simultaneously. Exactly how ADP alters the P_i–glucose-6-P response is unknown.

Results of the present investigation are in harmony with the model of brain hexokinase regulation described in Scheme I. There is no evidence from the initial-rate experiments of the back-reaction that glucose-6-P binds to a high-affinity allosteric site and to a low-affinity active site as has been suggested (Lazo et al., 1980).

Registry No. Glucose-6-P, 56-73-5; ADP, 58-64-0; P_i, 14265-44-2; MgATP, 1476-84-2; MgADP, 7384-99-8; hexokinase, 9001-51-8.

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One-Electron Reduction of D-Amino Acid Oxidase. Kinetics of Conversion from the Red Semiquinone to the Blue Semiquinone†

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ABSTRACT: The reduction of D-amino acid oxidase (DAAO) by hydrated electrons (e_{aq}^-) has been studied in the absence and presence of benzoate by pulse radiolysis. The e_{aq}^- did not reduce the flavin moiety in DAAO and reacted with the amino acid residues in the protein. In the presence of benzoate, e_{aq}^- first reacted with benzoate to yield benzoate anion radical. Subsequently, the benzoate anion radical transferred an electron to the complex of DAAO–benzoate to form the red semiquinone of the enzyme with a second-order rate constant of 1.2×10^9 M^{-1} s^{-1} at pH 8.3. After the first phase of the reduction, conversion of the red semiquinone to the blue semiquinone was observed in the presence of high concentration of benzoate. This process obeyed first-order kinetics, and the rate increased with an increase of the concentration of benzoate. In addition, the rate was found to be identical with that of the formation of the complex between benzoate and the red semiquinone of DAAO as measured by a stopped-flow method. This suggests that bound benzoate dissociates after the reduction of the benzoate–DAAO complex by benzoate anion radical and that free benzoate subsequently recombines with the red semiquinone of the enzyme to form the blue semiquinone.

It is well-known that a one-electron reduction of flavoproteins produces a stable free radical of the flavin molecule, which is produced by partial dithionite titration or photochemical processes in the presence of EDTA[1] (Massey & Palmer, 1966). These are protonated (the blue semiquinone) or unprotonated (the red semiquinone) in the physiological pH range. The pK value associated with these flavin radical species free in solution is 8.5 (Ehrenberg et al., 1967; Land & Swallow, 1969; Meisel & Neta, 1975). Most flavoproteins, however, form only blue or red semiquinones of the radical species independently of the external pH (Massey & Palmer, 1966; Massey et al., 1969) except glucose oxidase (Stankovich et al., 1978). This suggests that the particular protein to which the flavin is bound stabilizes either the blue or red semiquinone (Massey & Hemmerich, 1980). For example, the semiquinone of flavodoxin is the blue semiquinone even at a pH value above 10 (Mayhew & Massey, 1969; Mayhew, 1971; Edonondson & Tollin, 1971). Burnett et al. (1974) proposed that the blue semiquinone of flavodoxin is stabilized by a hydrogen bond between the N-5 H of the flavin radical and the carbonyl oxygen of glycine from X-ray analysis of the oxidized and semiquinone forms of flavodoxin. On the other hand, there are other interesting examples in which the ionization state of a flavin radical is influenced by the presence of a ligand (Massey & Palmer, 1966; Yasuda et al., 1967; Mizzer & Thorp, 1981; Yagi et al., 1972). For example, the semiquinone form of DAAO is the red semiquinone in the physiological pH range, whereas it converted into the blue semiquinone upon formation of the benzoate–enzyme complex (Yagi et al., 1972). Here, benzoate combines with DAAO at the substrate site (Yagi, 1962; Massey & Ganther, 1965). From this point of view, kinetic behavior of the semiquinone of flavin is expected to give interesting information about the environment of the flavin prosthetic group of these flavoproteins.

On the other hand, some of the advantages of the pulse radiolysis technique for determining the spectral and kinetic behavior of one-electron-reduction products of flavin have been demonstrated (Land & Swallow, 1969; Meisel & Neta, 1975; Faraggi et al., 1975). Recently this technique has been employed in the studies of flavodoxin (Faraggi & Klapper, 1979) and ferredoxin-NADP reductase (Maskiewicz & Bielski, 1982).

The present paper describes the reduction of DAAO by e_{aq}^- in the presence and absence of benzoate by the use of pulse radiolysis. We focus our attention on the semiquinone form of DAAO.

Materials and Methods

Hog kidney D-amino acid oxidase (DAAO) was purified by the procedure of Kubo et al. (1960) with the modifications of Massey et al. (1961) and Curti et al. (1973). The enzyme was prepared as the benzoate-bound form, and benzoate was freed from the enzyme, prior to each experiment, by passage of the enzyme, after reduction with excess D-alanine, through a column of Sephadex G-25 (Yagi & Ozawa, 1962a,b). Enzyme concentration was determined on the basis of FAD bound to the enzyme, using a molar extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm, and is expressed as subunit moles per liter. All other reagents were obtained commercially as the analytical grade.

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[1] Abbreviations: DAAO, D-amino acid oxidase; FAD, flavin adenine dinucleotide; e_{aq}^-, hydrated electron; EDTA, ethylenediaminetetraacetic acid.

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