Allosteric Activation of Brain Hexokinase by Magnesium Ions and by Magnesium Ion–Adenosine Triphosphate Complex

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1. Substrate-saturation curves of brain hexokinase for MgATP^{2-} were sigmoidal at sub-saturating concentrations of glucose when the $\text{Mg}^{2+}/\text{ATP}$ ratio was maintained at 1:1. Under identical conditions, except that Mg^{2+} was present in excess, hyperbolic curves were observed. 2. The number of binding sites (calculated from Hill plots) is 1.8 at a $\text{Mg}^{2+}/\text{ATP}$ ratio 1:1, and 1.0 with excess of Mg^{2+} . The apparent K_m for MgATP^{2-} is $6.5 \times 10^{-4} \text{ M}$ at a $\text{Mg}^{2+}/\text{ATP}$ ratio 1:1, and $3.5 \times 10^{-4} \text{ M}$ with excess of Mg^{2+} . 3. Interdependence between substrate-binding sites was indicated by the effects of varying the concentration of glucose. The sigmoidality and deviation from Michaelis–Menten kinetics at a $\text{Mg}^{2+}/\text{ATP}$ ratio 1:1 became less pronounced with increasing glucose concentration. Also, although substrate-saturation curves for glucose were hyperbolic when the $\text{Mg}^{2+}/\text{ATP}$ ratio was 1:1, reciprocal plots were non-linear. These were linear with excess of Mg^{2+} . 4. High concentrations of Mg^{2+} ($\text{Mg}^{2+}/\text{ATP}$ ratios above 5:1) were inhibitory. 5. The results are taken to indicate homotropic co-operative binding of MgATP^{2-} and that Mg^{2+} is an allosteric activator. Possible implications in regulation are discussed.

In a previous study of the involvement of Mg^{2+} and adenine nucleotides in regulation of mammalian cerebral-cortex hexokinase (EC 2.7.1.1) (Bachelard & Goldfarb, 1969), free ATP was shown to inhibit in a 'mixed' or partially competitive manner with respect to MgATP^{2-} . The inhibition by free ATP occurred when the $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratio was less than 1:1. A study of the uninhibited activity with $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of 1:1 and 2:1 showed a change in the substrate kinetic constants. At $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of 1:1, the apparent K_m for MgATP^{2-} was $6.5 \times 10^{-4} \text{ M}$; excess of Mg^{2+} ($\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of 2:1) gave a lower value of $3.5 \times 10^{-4} \text{ M}$ (Figs. 1 and 2 of Bachelard & Goldfarb, 1969). The enzyme not only showed this higher affinity for MgATP^{2-} in the presence of excess of Mg^{2+} but was also more active at limiting concentrations of MgATP^{2-} . This was not commented on at the time but retrospective analysis of the results indicated an activation by Mg^{2+} . The Dixon plots of that work ($1/v$ versus $[\text{ADP}]$) showed a tendency towards curvature at low $[\text{ADP}]$. Even though straight lines were drawn, subsequent scrutiny led to the view that the deviation from rectilinearity might not, as had been supposed, have been due to difficulty of distinguishing between reaction of free ADP^{3-} and MgADP^{-} , but could indicate allosteric interaction (Mahler & Cordes, 1966). This previous work was performed on the

cytoplasmic enzyme at saturating (2 mM) concentration of the second substrate, glucose. The above considerations made me decide to analyse the interaction of Mg^{2+} and ATP^{4-} more closely at limiting glucose concentration. A comparison of the purified solubilized particulate enzyme and the cytoplasmic enzyme from the brain has led to the conclusion that they are identical in chromatographic behaviour (Thompson & Bachelard, 1970) and in reaction mechanism (Bachelard, Clark & Thompson, 1971). In the present report, only the cytoplasmic hexokinase has been studied. The results indicate a homotropic co-operative effect of the substrate, MgATP^{2-} , and that Mg^{2+} acts as a positive allosteric effector when it is in excess ($\text{Mg}^{2+}/\text{ATP}^{4-} = 2:1$). However, high concentrations of Mg^{2+} are inhibitory.

MATERIALS AND METHODS

Hexokinase. The cytoplasmic hexokinase of guinea-pig brain, prepared as described for the ox brain enzyme (Bachelard & Goldfarb, 1969), was used and was assayed as follows. Measurements were made in duplicate at 37°C in semi-micro cuvettes in a Unicam SP. 800 recording spectrophotometer equipped with a constant-temperature cell housing. The incubation medium contained (final volume, 1 ml): 0.15 M -triethanolamine-HCl buffer, adjusted to pH 7.6 with KOH, 0.25 mM -NADP $^{+}$, glucose 6-phosphate dehydrogenase (0.07 international unit) and

glucose, Mg^{2+} and ATP^{4-} . Glucose concentrations were in the range 0.025–0.1 mM, Mg^{2+} 0.05–20 mM, and ATP 0.05–2 mM, as indicated in the text. Concentrations of Mg^{2+} were checked by atomic-absorption spectrometry, and those of ATP by spectrophotometry. After equilibration of the reaction mixtures for 5 min at 37°C, the assays were started by addition of hexokinase (usually 50 μl) and the formation of NADPH was followed by measuring E_{340} for at least 5 min and usually for 10 min. No change in E_{340} occurred during the equilibration period, nor when the final reaction mixture contained all constituents except glucose or ATP.

Reagents. Triethanolamine-HCl, the sodium salts of ATP and NADP^+ and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, type II purity from yeast) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were of the highest purity available from British Drug Houses Ltd., Poole, Dorset, U.K. Measurements of pH were made with a pH-meter (Radiometer, Copenhagen, Denmark) by using a glass electrode (type G 202 C).

RESULTS

Co-operative effects of MgATP^{2-} . The possibility that Mg^{2+} is involved in the hexokinase reaction in a fashion distinct from its role as MgATP^{2-} was explored by examining the initial velocity versus substrate concentration curves at $\text{Mg}^{2+}/\text{ATP}^{4-} =$

1:1 and $\text{Mg}^{2+}/\text{ATP}^{4-} = 5:1$ (Fig. 1). When Mg^{2+} was maintained in excess (Fig. 1a), hyperbolic curves were obtained at all glucose concentrations tested, which included limiting concentrations of 0.025–0.1 mM and also saturating concentrations of 2 mM. Fig. 1(a) shows the results observed with $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of 5:1; similar curves were obtained with ratios of 2:1. However, when the $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratio was maintained at 1:1, curves of distinctly different shape, suggesting a sigmoidal character, were observed (Fig. 1b). In contrast also to the curves in Fig. 1(a) (excess of Mg^{2+}), where saturation was approached at 0.8 mM- MgATP^{2-} , the rates in Fig. 1(b) were still increasing at that concentration. The involvement of glucose was also noteworthy: the sigmoid character of the curves became more pronounced as the glucose concentration decreased from 0.1 mM to 0.025 mM. Moreover, although excess of Mg^{2+} caused some activation at saturating glucose concentration [the results of Figs. 1, 2 and 3 of Bachelard & Goldfarb (1969) were confirmed in the present study] and at the lower MgATP^{2-} concentrations of the present work, no activation by Mg^{2+} was detected at the higher concentrations (0.4–0.8 mM) of MgATP^{2-} and the limiting glucose concentrations in Fig. 1. The two sets of curves in Fig. 1 are strictly comparable, having

Fig. 1. MgATP^{2-} saturation curves for brain hexokinase at $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of (a) 5:1 and (b) 1:1, with glucose concentrations of 0.1 mM ($\circ$), 0.05 mM ($\bullet$), 0.035 mM ($\square$) and 0.025 mM ($\blacksquare$). Experimental details are given in the Materials and Methods section.

been obtained by 'randomization' of experimental series with all constituents identical except Mg^{2+} . The curves in Fig. 1(b) are significantly different from the companion curves in Fig. 1(a), at MgATP^{2-} concentrations up to 0.3 mM with 0.1 mM-glucose, and up to 0.15 mM with the lower glucose concentrations used. The s.d. of the measurements, observed usually over 10 min, was ± 0.001 or ± 0.002 $\Delta E/\text{min}$, depending on the rates.

The departure from Michaelis-Menten kinetics when $\text{Mg}^{2+}/\text{ATP}^{4-} = 1:1$ is shown more clearly in Fig. 2: plots of $[\text{MgATP}^{2-}]/v$ versus $[\text{MgATP}^{2-}]$ were linear in the presence of excess of Mg^{2+} (Fig. 2a), but non-linear when $\text{Mg}^{2+}/\text{ATP}^{4-}$ was maintained at 1:1 (Fig. 2b). Plots of $1/v$ versus $1/[\text{MgATP}^{2-}]$ (not shown) were also curved when $\text{Mg}^{2+}/\text{ATP}^{4-}$ was 1:1. Again, the non-linearity became less pronounced at the higher glucose concentrations and disappeared at saturating glucose

(see also Fig. 2 of Bachelard & Goldfarb, 1969). The kinetic plots with respect to glucose as varied substrate were also different: plots of $[\text{glucose}]/v$ versus $[\text{glucose}]$ were linear when Mg^{2+} was in excess (Fig. 3a) but curved when $\text{Mg}^{2+}/\text{ATP}^{4-}$ was 1:1 (Fig. 3b).

Effect of Mg^{2+} on MgATP^{2-} -binding sites. The slopes of Hill plots $\{\log[v/(V-v)]\}$ versus $\log[\text{MgATP}^{2-}]$ (Hill, 1910; Atkinson, Hathaway & Smith, 1965) give a measure of n , the number of substrate-binding sites per molecule. Those obtained when 0.1 mM-glucose was used are shown as the insets to Fig. 2. When $\text{Mg}^{2+}/\text{ATP}^{4-}$ was 5:1 (inset to Fig. 2a), $\bar{n} = 1.05$; when the ratio was 1:1 (inset to Fig. 2b), $\bar{n} = 1.8$. The lines of the Hill plots were drawn by the least-squares method. The points had correlation coefficients (r) to the lines of 0.997 (Fig. 2a) and 0.985 (Fig. 2b). The slopes of the lines in Figs. 2(a) and 2(b) were significantly different

Fig. 2. Plots of $[\text{MgATP}^{2-}]/v$ versus $[\text{MgATP}^{2-}]$ for brain hexokinase at $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of (a) 5:1 and (b) 1:1, with glucose concentrations of 0.1 mM ($\circ$), 0.05 mM ($\bullet$), 0.035 mM ($\square$) and 0.025 mM ($\blacksquare$), where v is expressed as $\Delta E_{340}/\text{min}$. The Hill plots (see the text), drawn from the results when 0.1 mM-glucose was present, appear as insets.

Fig. 3. Plots of $[\text{glucose}]/v$ versus $[\text{glucose}]$ for brain hexokinase at $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of (a) 5:1 and (b) 1:1, with MgATP^{2-} concentrations of 0.6 mM (○), 0.4 mM (●), 0.3 mM (□), 0.2 mM (■), 0.15 mM (▽) and 0.1 mM (▼), where v is expressed as $\Delta E_{340}/\text{min}$.

($P < 0.001$). The value for $\bar{n}$ varied also with glucose concentration when $\text{Mg}^{2+}/\text{ATP}^{4-}$ was maintained at 1:1 (Table 1); there was a gradual slight decrease in $\bar{n}$ for MgATP^{2-} as glucose concentrations decreased. All were significantly different from those obtained at $\text{Mg}^{2+}/\text{ATP}^{4-} = 5:1$, which did not vary significantly with changes in glucose concentration.

Plots of v versus $[\text{glucose}]$ (not shown) gave no indication of a sigmoidal character, even though the reciprocal plots were curved at $\text{Mg}^{2+}/\text{ATP}^{4-} = 1:1$ (Fig. 3b). Hill plots $\{\log[v/(V-v)]\}$ versus $\log[\text{glucose}]\}$ gave $\bar{n}$ values of 0.8–1.0; no significant difference was found to be due to Mg^{2+} .

Inhibition by excess of Mg^{2+} . Investigation of the effects of increasing the Mg^{2+} concentration up to an excess over ATP^{4-} of 10 mM showed that relatively large excess concentrations of Mg^{2+} caused slight inhibition (Fig. 4). The inhibition appeared to be more marked at 0.5 mM- than at 1.0 mM- MgATP^{2-} . The amounts of MgATP^{2-}

Table 1. Number ($\bar{n}$) of MgATP^{2-} -binding sites of hexokinase

These were measured at $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios of 1:1 and 5:1, derived from the slopes of Hill plots $\{\log[v/(V-v)]\}$ versus $\log[\text{MgATP}^{2-}]\}$. Results were significantly different ($*P < 0.001$, $\dagger P < 0.005$) from the values when $\text{Mg}^{2+}/\text{ATP}^{4-} = 5:1$ (see the text).

Glucose (mm)	$\bar{n}$	
	$\text{Mg}^{2+}/\text{ATP}^{4-} = 1:1$	$\text{Mg}^{2+}/\text{ATP}^{4-} = 5:1$
2.0	1.8†	—
0.1	1.8*	1.05
0.05	1.65*	1.1
0.035	1.5†	1.05
0.025	1.4†	1.05

† Calculated from the results in Fig. 2 of Bachelard & Goldfarb (1969).

present were calculated from the binding constant, 73000 M^{-1} (O'Sullivan & Perrin, 1964).

Fig. 4. Inhibition of brain hexokinase by large excess of Mg^{2+} . Dixon plot ($1/v$ versus $[I]$) where $[I]$ is the concentration of free Mg^{2+} . Calculated concentrations of MgATP^{2-} were 0.5 mM (○) and 1.0 mM (●). Glucose was present at 0.5 mM.

DISCUSSION

Co-operative effects of MgATP^{2-} . The change in substrate-saturation curves from sigmoidal ($\text{Mg}^{2+}/\text{ATP}^{4-} = 1:1$) to hyperbolic ($\text{Mg}^{2+}/\text{ATP}^{4-}$ ratio greater than 1) is typical of a co-operative interaction abolished by the presence of an allosteric activator according to the model proposed by Monod, Wyman & Changeux (1965). In this case, the allosteric activator is Mg^{2+} . The homotropic co-operative binding of MgATP^{2-} in the absence of excess of Mg^{2+} indicates that the substrate binds at more than one site on the enzyme, which is supported by the observation from the Hill plots of 2 molecules of substrate bound ($\bar{n} = 1.8$) in the absence of excess of Mg^{2+} . In the presence of excess of Mg^{2+} , one molecule of MgATP^{2-} only appears to be bound ($\bar{n} = 1$).

According to the model proposed by Atkinson *et al.* (1965), a positive effector such as Mg^{2+} would be expected to facilitate binding of MgATP^{2-} by altering the conformation of the protein, whereas the model of Monod *et al.* (1965) predicts that Mg^{2+} should decrease the homotropic interaction of MgATP^{2-} . The observations in the present work are not clearly in accordance with either of the two models. At the sub-saturating glucose concentrations in Figs. 1–3, excess of Mg^{2+} did not yield increased enzymic rates except at the lower range of MgATP^{2-} concentration; at the higher MgATP^{2-} concentrations, slight inhibition occurred (see also Fig. 4). However, at saturating glucose concentrations, definite activation occurs over the whole range of MgATP^{2-} concentrations tested. Thus, depending on which range of glucose concentration were to be chosen for study, the effects of Mg^{2+} could appear to be in support of one or other of the two allosteric models. If, as the

results suggest, there are two independent substrate-binding sites for MgATP^{2-} , with Mg^{2+} reacting at an effector site to alter the conformation of MgATP^{2-} -binding sites, it seems to be indicated that the glucose-binding site is also involved. Alterations in conformation of the MgATP^{2-} -binding sites may also affect the conformation of the glucose-binding site. The graduated effects of glucose on the MgATP^{2-} -saturation curves in Fig. 1(b) and on the reciprocal plots and Hill plots in Fig. 2(b) and Table 1 would suggest this to be the case.

The inhibition by high concentrations of free Mg^{2+} (Fig. 4) is not likely to be significant *in vivo*, as it became marked at $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios above 5:1 and at the lower MgATP^{2-} concentration tested. The Mg^{2+} concentrations involved are considerably greater than those likely to occur *in situ* (Bachelard & Goldfarb, 1969).

The hexokinase molecule would therefore appear to be more complex than has been suspected hitherto. In addition to the possibility of separate sites for free ATP, MgATP^{2-} and MgADP^{3-} (Bachelard & Goldfarb, 1969) and also for glucose and glucose 6-phosphate, there is now evidence for more than one MgATP^{2-} site and for a separate effector site for Mg^{2+} . Some of these, for example the MgATP^{2-} sites, the Mg^{2+} site and the glucose site may be interdependent, as shown by the effect of Mg^{2+} on the homotropic co-operative nature of the binding of MgATP^{2-} and the involvement of glucose in this binding.

The enzyme may therefore contain as many as eight distinct sites: two for MgATP^{2-} , one for each of free ATP, Mg^{2+} , MgADP^{3-} and/or ADP^{3-} , glucose and glucose 6-phosphate.

Allosteric properties of brain hexokinase have previously been indicated by the non-competitive nature of the inhibition by free ATP and by observations of non-competitive inhibition by its products (glucose 6-phosphate and ADP^{3-}). Such observations have not been consistent, however. Competitive inhibition by glucose 6-phosphate with respect to ATP^{4-} has been reported (Fromm & Zewe, 1962) whereas Newsholme, Rolleston & Taylor (1968) found it to be non-competitive. Similarly, inhibition by ADP^{3-} has been found competitive for MgATP^{2-} by some workers (Sols & Crane, 1954), but non-competitive by others (Fromm & Zewe, 1962; Bachelard & Goldfarb, 1969). It seems possible that the different observations may have been caused by variations in the $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratios used (see Newsholme *et al.* 1968; Bachelard & Goldfarb, 1969; Kosow & Rose, 1970). If, as the present work suggests, allosteric properties are present when Mg^{2+} is not in excess and are abolished by excess of Mg^{2+} , allosteric product inhibition may not be observed so readily

in the presence of excess of Mg^{2+} . It also seems feasible that the allosteric behaviour may be less obvious at saturating glucose concentration.

Hexokinase has long been considered to be one of the key control points for regulation of cerebral carbohydrate metabolism (Lowry, Passonneau, Hasselberger & Schulz, 1964; Bachelard, 1970) and the present evidence of allosterism gives support to this. The effector role of Mg^{2+} is noteworthy in attempts to assess the regulation of the enzymic activity in terms of intracellular concentrations of Mg^{2+} and of ATP^{4-} available *in vivo*. Without exact knowledge of these factors (Bachelard & Goldfarb, 1969) it is difficult to make precise predictions, but clearly, if the concentration of available ATP^{4-} fell below that of Mg^{2+} , the effector role of the Mg^{2+} could operate to activate the enzyme by facilitation of $MgATP^{2-}$ binding. In the presence of excess of ATP^{4-} , resulting from intracellular rates of ATP^{4-} formation exceeding those of ATP^{4-} utilization, it could be argued that the activity of the enzyme would be likely to decrease, not only by ATP^{4-} inhibition, but also by the lack of free Mg^{2+} for allosteric activation.

Similar but not identical evidence for allosteric involvement of Mg^{2+} and $MgATP^{2-}$ has been found for other $MgATP^{2-}$ -requiring enzymes, e.g. the pyruvate carboxylase of liver and kidney (Scrutton & Utter, 1967; Keech & Barritt, 1967). The properties of the cerebral hexokinase may be more complex: glucose does not itself show co-operative binding but affects the co-operative binding of $MgATP^{2-}$ in a concentration-dependent fashion. Thus the substrate sites may be inter-dependent. A closer examination of the involvement of glucose in causing conformational change is indicated in terms of the different theoretical

models proposed by Koshland, Nemethy & Filmer (1966).

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