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Biochemistry

Proton-dependent inhibition of yeast and brain hexokinases by aluminum in ATP preparations

(citrate activation/negative cooperativity/dialysis encephalopathy)

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ABSTRACT **The aluminum present as a contaminant in ATP preparations can cause strong inhibition of yeast hexokinase P-II activity at pH 7.0 or below but has little or no inhibitory effect at a pH of 7.5 or greater. The inhibition is reversed by citrate, 3-phosphoglycerate, malate, phosphate, and catecholamines, all of which have previously been described as activators of hexokinase at low pH. We suggest that these agents activate the enzyme only by virtue of their ability to coordinate with aluminum present in the assay system. The presence of aluminum is also responsible for the "negative cooperativity" observed at low pH with respect to Mg·ATP concentration—i.e., the inhibition by aluminum is uncompetitive at low Mg·ATP concentrations but becomes competitive at high Mg·ATP concentrations. The inhibition is thought to be due to formation of a complex of Al·ATP with the enzyme, with a dissociation constant (K_i) of 0.1 μM. Yeast hexokinase P-I is somewhat less sensitive to Al than is hexokinase P-II, and yeast glucokinase is not detectably affected. The hexokinase in rat brain (type I) shows a pH-dependent inhibition by Al similar to that observed with the yeast hexokinases, whereas the rat muscle (type II) enzyme is less sensitive, suggesting a possible relationship to aluminum encephalopathy in man.**

Kosow and Rose (1) showed that, when yeast hexokinase P-II was inhibited by lowering the pH from the optimum of 8.0 to values around 7.0 or below, it could be activated either by raising the Mg·ATP concentration ("substrate activation" or "negative cooperativity") or by adding certain metabolites—e.g., citrate, 3-phosphoglycerate, malate, or phosphate. We reported (see refs. 2 and 3, compare with ref. 4) that the inhibition by protons was not seen with certain buffers at pH 6.9, but that inhibition was then obtained by adding low concentration of ADP or GDP, and that this inhibition was reversed by any of the activators listed above.

We now report that the degree of inhibition of yeast hexokinase by protons is dependent on the aluminum content of the ATP (or ADP) used, and that the activation by excess Mg·ATP or by the above-mentioned metabolites is due to their ability to reverse inhibition by aluminum. We also report a similar sensitivity of brain hexokinase to aluminum and suggest a possible relation to cases of encephalopathy associated with aluminum toxicity in man.

MATERIALS AND METHODS

Yeast hexokinase isozymes P-I and P-II were prepared as described (5). They were dialyzed vs. 50 mM potassium phosphate, pH 7/0.1 mM EDTA and stored frozen as concentrated solutions of approximately 20 mg of protein per ml. These frozen stocks were diluted 1:1000 each day in 50 mM Tris·HCl, pH 6.9, containing 0.1 mM EDTA. From 0.005 to 0.03 units was added to reaction mixtures in 2- to 4-μl volumes. The specific activity of the stock P-II was 650 units/mg and that of the stock P-I was 160 units/mg.

Glucose-6-phosphate dehydrogenase, purity grade I, was purchased from Boehringer Mannheim. It was dialyzed vs. 50 mM Tris·HCl, pH 6.9/0.1 mM EDTA and stored frozen. Approximately 1 unit was added to each reaction in a volume of 2 μl.

The hexokinase reaction was coupled to the glucose-6-phosphate dehydrogenase reaction in the enzyme activity measurements. Reaction mixtures contained 1.0 ml of a stock solution [50 mM imidazole/1 mM glucose/0.22 mM NADP/5 mM $MgCl_2$/0.1 mM EDTA (unless otherwise stated)] and glucose-6-phosphate dehydrogenase. ATP or Al·ATP was added as indicated. Reactions were begun by the addition of hexokinase and measured by the increase in absorbance at 340 nm for 1–9 min.

The data presented for the yeast hexokinases are for the increase in absorbance for 1 min, commencing 20 sec after hexokinase addition. The effects reported are therefore those on the initial "burst" (6), but very similar effects of aluminum were seen on the slower steady-state rate.

The hexokinase in lyophilized rat brain or muscle was purified by DEAE-cellulose chromatography according to Grossbard and Schimke (7) and the peak fractions of type I enzyme (brain) or type II enzyme (muscle) were pooled. The pooled material was concentrated 25-fold by ultrafiltration in a Minicon-B-15 Amicon Concentrator. About 4 μl of concentrate containing 0.005–0.015 units of hexokinase were used in each assay. The final concentration of phosphate (20 μM) introduced into the reaction mixture with the hexokinase was insufficient to interfere appreciably with the inhibition by aluminum. There was no "burst" observed in the brain or muscle enzyme assays. In fact, there was usually a slight lag during the first minute. The data reported are for the maximal rates observed after this lag period.

RESULTS

Inhibition of Yeast Hexokinase by a Metal in ATP Preparations. Initial evidence for an inhibitory metal in certain ATP preparations came from kinetic studies at pH 6.9 in which the hexokinase reaction was started by adding ATP as the last component to an otherwise complete system containing 1 mM EDTA. With some ATP preparations, a very low initial rate was seen, but activity increased strikingly (about 10-fold) during a 6-min assay period. The increase with time was seen only when 1 mM EDTA was present. It was then found that the ATP itself could be "activated" by simply allowing it to stand with 1 mM EDTA in the buffer for the same 6-min period prior to starting the reaction with enzyme. This is illustrated in Fig. 1, in which the results with two different disodium ATP samples are shown. Lot 51001 from P-L Biochemicals showed very little activity unless preincubated with EDTA, whereas lot 81014 from the same company was almost fully active without such

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

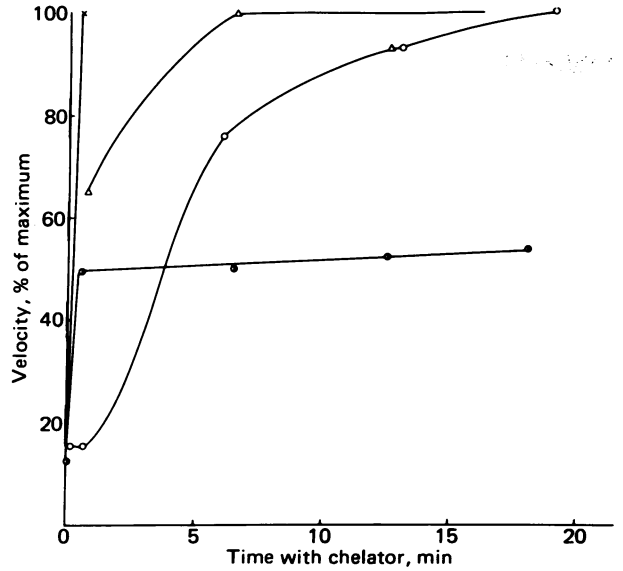

FIG. 1. Effect of time of incubation of ATP with metal chelators prior to addition of yeast hexokinase. Reaction mixtures (pH 6.9) containing all standard components plus the indicated chelators, but no hexokinase, were kept at 25°C for various times, as measured from the time of initial contact of ATP with chelator, and the reaction was started by addition of yeast hexokinase P-II. △, 0.5 mM ATP (P-L Biochemicals lot 81014)/1 mM EDTA; ○, 0.5 mM ATP (P-L Biochemicals lot 51001)/1 mM EDTA; ◑, 0.5 mM ATP (P-L Biochemicals lot 51001)/1 mM EGTA; ×, 0.5 mM ATP (P-L Biochemicals lot 51001)/0.5 mM citrate.

incubation. Other preparations (e.g., disodium ATP from equine muscle; Sigma) gave intermediate results. Ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) caused a much more rapid "activation" of the ATP than did EDTA, but the extent of activation was smaller (Fig. 1). These findings, together with the observation that the low-activity ATP preparation was instantly brought to full activity at pH 6.9 by addition of citrate (Fig. 1) led us to believe that the phenomenon of citrate activation might be due simply to reversal of inhibition by a metal contaminant in the ATP. This metal apparently caused inhibition only at low pH, because the two ATP preparations were both fully active in the pH 8 assay without addition of any activator.

Inhibitory Metal Is Aluminum. Semiquantitative metal analyses by emission spectroscopy (Stewart Labs, Knoxville, TN) showed that, of 37 metals determined, only aluminum was higher in lot 51001 than in lot 81014 (0.04 vs. 0.003%). The only other detectable metal in lot 51001 was Fe (0.004%), whereas lot 81014 contained measurable Fe (0.01%), Ba (0.001%), Ca (0.005%), and Mn (0.04%). Subsequent quantitative atomic absorption analysis for aluminum gave values of 0.033% and 0.002% for lots 51001 and 81014, respectively. These values correspond approximately to a ratio of 1 Al atom to 100 ATP molecules in lot 51001 and 1 Al atom to 1600 ATP molecules in lot 81014. Thus, a 0.5 mM solution of ATP from lot 51001 (the usual concentration for studying activator effects) would contain about 5 μM Al^{3+}, whereas the same concentration of ATP from lot 81014 would contain only about 0.3 μM Al^{3+}.

When sufficient $AlCl_3$ was added to an ATP stock solution from lot 81014 to bring its Al content up to that of ATP from lot 51001, the expected degree of inhibition was observed. (Fig. 2*A*). The presence of 0.4 μM total Al^{3+} in the reaction mixture with 0.5 mM ATP caused about half-maximal inhibition of yeast hexokinase P-II at pH 6.9. It should be noted that it is necessary to add the $AlCl_3$ to the ATP stock solution in order to observe the effects illustrated here. When the same amounts of Al^{3+} are added to the reaction mixtures separately from the ATP, more than 10 times as much Al^{3+} is needed to get a comparable effect.

Fig. 2*A* also shows that yeast hexokinase P-I is less sensitive to Al^{3+} than is P-II, requiring about 1.8 μM total Al for half-maximal effect at 0.5 mM ATP. This result is compatible with the previous finding (3) that hexokinase P-I is less susceptible to inhibition by protons than is P-II.

The concentrations of Al^{3+} required for inhibition of P-I and P-II were even lower when lower concentrations of ATP were used. With 0.1 mM ATP (Fig. 2*B*), 0.2 μM Al^{3+} was sufficient for half-maximal inhibition of P-II.

pH-Dependence of Al^{3+} Inhibition. When the effect of Al^{3+} was tested on yeast hexokinase P-II at various pH values, a marked increase in sensitivity to Al^{3+} was observed as the pH was lowered (Fig. 3). Expressed in another way, the addition

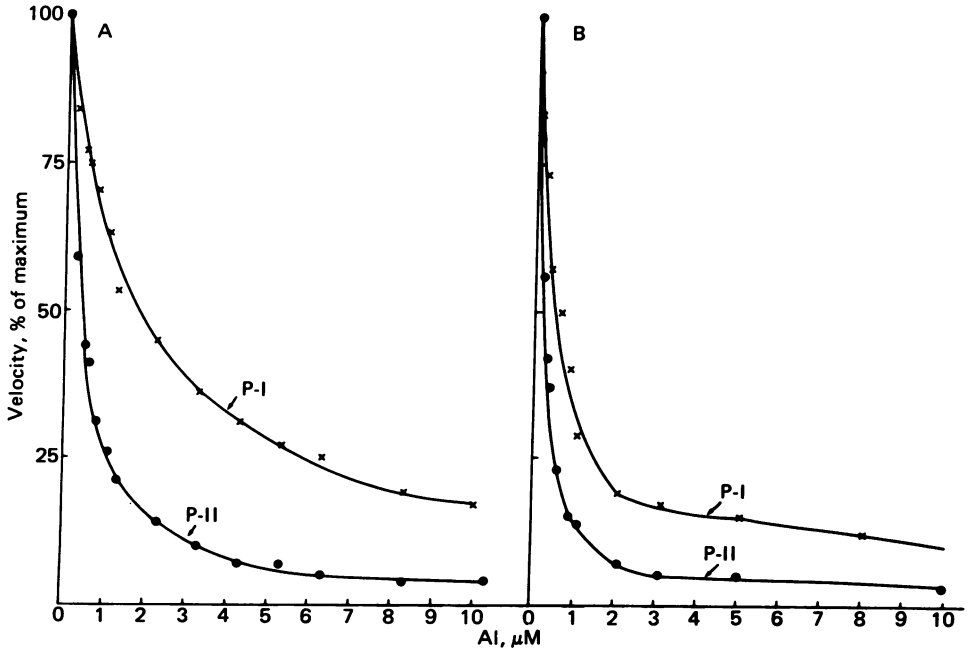

FIG. 2. Effect of varying aluminum content of ATP on activity of yeast hexokinases P-I and P-II. The reaction mixtures (pH 6.9) were prepared as described but contained no EDTA. The ATP samples of varying aluminum content were prepared by adding small volumes of 1–100 mM $AlCl_3$ (Fisher) to stock solutions of 10 or 100 mM disodium ATP (lot 81014) that had been adjusted previously with NaOH to ≈pH 6–6.5. Aliquots of these "pre-mixed" Al·ATP stocks were then added to the 1-ml reaction mixture in the cuvette, and the reaction was started within 30 sec by the addition of hexokinase. The final ATP concentration was 0.5 mM (*A*) or 0.1 mM (*B*). The rates shown at zero Al^{3+} concentration are those with 0.5 mM citrate added. The other Al^{3+} concentrations shown are calculated to include the amount contributed by the aluminum in the ATP preparation.

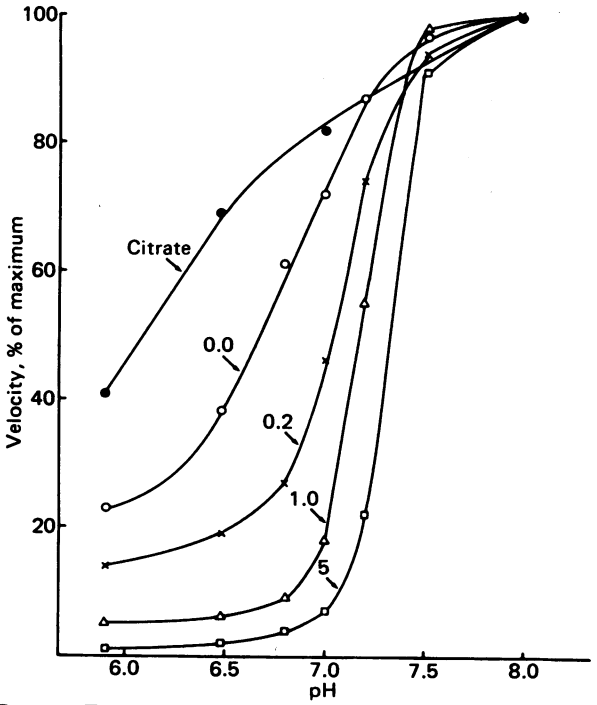

FIG. 3. Effect of pH on aluminum inhibition of yeast hexokinase P-II. Reaction mixtures were prepared as described, and the pH was adjusted to values noted. ATP (lot 81014) was premixed with $AlCl_3$ in various concentrations. The final ATP concentration in the reactions was 0.1 mM. The numbers beside the data curves give the final μM concentration of added aluminum in the reactions. The reaction was begun by the addition of P-II within 30 sec after the addition of Al·ATP. No aluminum was added to the reactions denoted by the "citrate" curve; 0.5 mM sodium citrate was present in this reaction mixture.

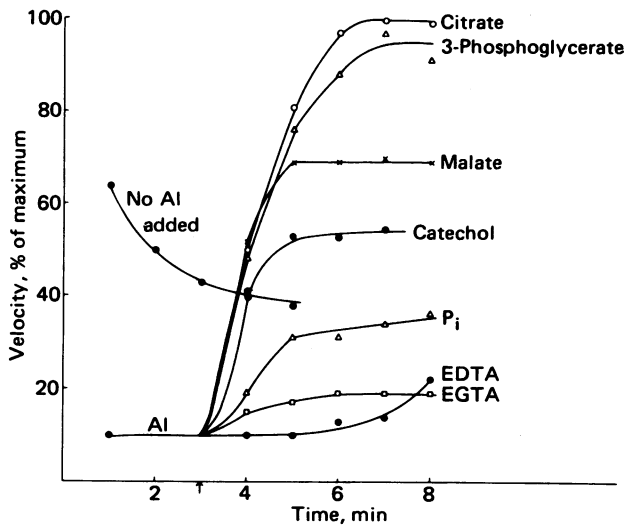

FIG. 4. Reversal of aluminum inhibition of yeast hexokinase P-II by citrate and other agents. Reaction mixtures were prepared without EDTA at pH 6.9. ATP (lot 81014) was premixed with $AlCl_3$. Final ATP concentration was 0.5 mM, and Al^{3+} was 5 μM. The Al·ATP was added to the reaction mixture, and the reaction was started by the addition of P-II within 20 sec. Effectors were added at 3 min. The rate of the reaction is shown as a function of time. The control with no Al^{3+} added actually contained 0.3 μM Al^{3+} derived from the ATP preparation. Effector concentrations (mM): citrate, 0.5; 3-phosphoglycerate, 0.5; malate, 1.0; catechol, 0.5; P_i, 1.0; EDTA, 1.0; EGTA, 1.0.

of Al^{3+} made the system much more sensitive to inhibition by protons, shifting the apparent pK_a from a value around 6.7 (in the sample without Al added) to values around 7.1, 7.2, and 7.3 in the samples with 0.2, 1, and 5 μM $AlCl_3$ added, respectively. The ATP sample at the concentration used (0.1 mM in this experiment) contained 0.06 μM Al^{3+}. We assume that this concentration of Al^{3+} is sufficient to cause the pK shift from 6.1 (citrate sample) to 6.7. In the original experiments of Kosow and Rose (1), they found an apparent pK_a of 7.3 for this enzyme system; this suggests to us that their test system may have contained around 5 μM Al^{3+}.

In addition to shifting the apparent pK_a for enzyme activity, aluminum causes a change in the stoichiometry of reaction of the enzyme with protons. Kosow and Rose (1) have noted that, in the absence of activators, the activity fell very steeply with proton concentration, and from a Hill-type plot concluded that two protons were involved in conversion of the active enzyme to an inactive form. In the present experiments, the samples to which Al^{3+} was added (0.2–5 μM) also showed a steep fall in activity with proton concentration in the pH range 7.0 to 7.5, corresponding to around three protons per molecule of enzyme inactivated. By contrast, the citrate-containing sample gave a value of only 0.66 proton per molecule.

Reversal of Al^{3+} Inhibition. Fig. 4 shows that the inhibitory effect of added $AlCl_3$ is reversed by subsequent addition of any of the various substances previously reported to be activators of the enzyme, including citrate, 3-phosphoglycerate, phosphate, and malate (1), as well as catechol (8). The citrate effect corresponds closely in rate of onset and extent to that seen upon addition of citrate to test systems containing the inhibitory ATP from lot 51001. It seems probable that the various activators function by virtue of their ability to form coordination complexes with aluminum. It should be noted that under the conditions in Fig. 4, where enzyme inhibition by Al^{3+} has occurred prior to addition of activator, reversal requires much more time than for "activation" of Al·ATP (Fig. 1). This difference is especially striking in the case of EDTA (1 mM), which shows very little activation under the conditions in Fig. 4. EDTA at 0.1 mM did not appreciably affect Al^{3+} inhibition under assay conditions and could therefore be included in reaction mixtures to protect against inhibition by heavy metal ions.

The control in Fig. 4 with no Al added shows the burst of activity typical of the yeast enzyme in the low pH region (4). Al^{3+} inhibited both the burst and the subsequent slower phase of the reaction. Citrate addition to the inhibited system, as in Fig. 4, restored activity but did not restore the burst. However, when citrate was used to prevent inhibition, as in Fig. 1, the burst effect was fully retained (data not shown).

The Al^{3+} inhibition could also be reversed by raising the concentration of Mg·ATP to sufficiently high levels (Fig. 5). It can be seen that the Al^{3+}-inhibited system exhibits "negative cooperativity" with respect to Mg·ATP concentration, much like the kinetics originally reported by Kosow and Rose (1) for the proton-inhibited system. The system to which no Al was added showed some apparent substrate inhibition at high Mg·ATP concentrations (0.5 mM and above). This is probably due to the presence of traces of Al^{3+}, even in the low-Al preparation used. In support of this view, citrate can be shown to overcome this "substrate inhibition". This kind of inhibition was minimized in those experiments in which we used 0.1 mM ATP instead of 0.5 mM ATP (e.g., in Figs. 2*B* and 3).

Effect of Aluminum on Mammalian Hexokinases. Because type I mammalian hexokinase, the predominant form in the brain, has been shown to be stimulated by catecholamines (9) and by citrate (10), it appeared likely that it would share with the yeast hexokinases a sensitivity to Al^{3+}. In initial experiments with homogenates of mouse brain, 1 μM added Al^{3+} caused about 50% inhibition under conditions similar to those in Fig. 2*A* (Thomas Rand, personal communication). When a partially purified type I rat brain enzyme was used, about 2 μM added

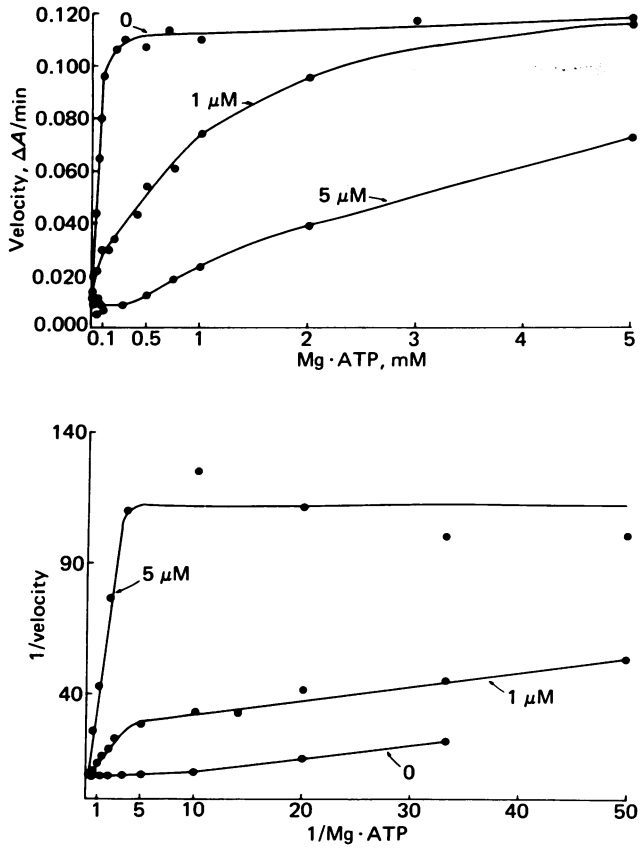

FIG. 5. Apparent "negative cooperativity" with respect to Mg·ATP due to aluminum. Reaction mixtures were prepared at pH 6.9. $AlCl_3$ was premixed with ATP lot 81014 for two sets of experiments: one to give a 1 μM final concentration of added Al^{3+} and the other to give a 5 μM final concentration of added Al^{3+}. In the control experiment, no $AlCl_3$ was premixed with the ATP. The reactions were begun by the addition of yeast hexokinase P-II within 20 sec after the addition of ATP or Al·ATP. The concentration of $MgCl_2$ was in 5 mM excess of the ATP concentration.

Al^{3+} caused 50% inhibition (Fig. 6A). As with the yeast enzymes, the Al^{3+} must be premixed with the ATP to get the degree of inhibition shown here. The brain enzyme showed the same strong pH dependence as yeast hexokinase for inhibition by Al^{3+} (Fig. 6B). In the presence of 5 μM Al^{3+}, the pK_a was about 7.3 and the slope of the Hill plot (not shown) was close to 3. Inhibition by endogenous or added Al^{3+} was fully prevented by citrate. When a crude rat brain homogenate was used instead of the partially purified enzyme, the results were almost identical with those in Fig. 6.

Type II mammalian hexokinase, the predominant form in muscle, is not stimulated by citrate or catecholamines (10). Correspondingly, the type II enzyme from rat muscle was found to be relatively insensitive to Al^{3+} (Fig. 6A), requiring around 10 μM Al^{3+} for 50% inhibition.

Specificity of Aluminum Effect. When various trivalent metal ions were tested for their effects on yeast hexokinase P-II in imidazole buffer at pH 6.9 in the presence of 0.1 mM ATP, no effect was found with Ga^{3+} [10 μM $Ga(ClO_4)_3$; Alfa Inorganics (Beverly, MA)], Cr^{3+} [10 μM $Cr(C_2H_3O_2)_3$; Fisher], or Fe^{3+} (100 μM $FeCl_3$). These metals were tested by prior addition to the ATP stock solution, so that conditions were exactly comparable to those under which inhibition by Al^{3+} was observed. Boron (5 μM $Na_2B_4O_7$) was also without effect in this system. Sodium ortho- or meta-vanadate (V^{5+}) at a concentration of 2 mM caused 50% inhibition of P-I or P-II. Vanadyl sulfate [$VOSO_4$ (V^{4+}; gift of Robert Post)] caused complete inhibition of P-I or P-II at 0.4 mM but little or no inhibition at 0.04 mM. Citrate had little or no activating effect on the vanadium-inhibited systems.

Al^{3+} inhibition shows a specificity for hexokinases. When yeast glucokinase (11) from *Saccharomyces cerevisiae* was separated from hexokinases P-I and P-II on a hydroxyapatite column (12), it showed no inhibition by 5 μM Al^{3+} at pH 6.9. When rabbit phosphofructokinase was assayed at pH 6.9 at either optimal (0.1 mM) or inhibitory (0.5 mM) ATP concentrations (13), there was no effect of 10 μM Al^{3+} on the activity.

DISCUSSION

It seems clear from these studies that the apparent regulatory effects of citrate and other substances on yeast (or brain) hexokinase at low pH are seen only when the enzyme is inhibited by traces of aluminum in the test system, so that activation is due simply to chelation of Al^{3+}. This finding accounts for our previous failure to detect citrate binding to yeast hexokinase P-II or S-II (3). It remains to be seen whether regulation of hexokinases by chelation of Al^{3+} has any physiological significance.

In the present case, the aluminum was introduced as a contaminant in the ATP. In some previous reports from our laboratory (3) in which ADP and GDP were said to produce inhibitions that were citrate-reversible, it seems clear now that the effects were due to Al contamination. Two ADP samples, identical in their inhibitory power, showed 0.010 and 0.011% Al by weight by atomic absorption analysis. A GDP sample that was noninhibitory (in disagreement with earlier results) proved to be low in Al (0.002%).

Kosow and Rose (1) had considered the possibility that the activation by citrate and other substances might be secondary to contamination of the system by inhibitory metals. They tested a large series of divalent metals and found that none of them inhibited the enzyme sufficiently to account for the observed citrate effect. As a further precaution, they had treated all components of the system by shaking with a solution of 0.1 M 8-hydroxyquinoline in chloroform. We have found that such treatment fails to remove appreciable Al^{3+} from a stock solution containing 0.1 M sodium ATP and 1 mM $AlCl_3$ at pH around 6.4, although the same treatment was fully effective in removing the Al^{3+} from 0.1 M imidazole, pH 6.9/1 mM $AlCl_3$. Thus ATP appears to be a better chelator for Al^{3+} than is 8-hydroxyquinoline and was possibly a source of contamination with Al in the Kosow–Rose experiments.

The Al^{3+} appears to exert its effect as a complex with ATP. We assume this from the following evidence. (*i*) Al^{3+} is much more inhibitory (over 10-fold) when premixed with the 0.1 M stock solution of disodium ATP than when added to the final reaction mixture separately from the ATP. (*ii*) When the Mg·ATP concentration in the assay system is increased, one sees two effects: (*a*) in the low Mg·ATP concentration range (0–0.2 mM), the percentage inhibition by Al^{3+} increases as the Mg·ATP is raised, and (*b*) in the higher Mg·ATP range (0.2–5.0 mM), the percentage inhibition by Al^{3+} decreases as the Mg·ATP is raised (Fig. 5). This could mean that Al^{3+} forms an inhibitory complex with ATP and that this Al·ATP complex competes with Mg·ATP for the active site of the enzyme. From the data in Fig. 5 for the high Mg·ATP concentration range, one can estimate that the K_i for the presumed Al·ATP complex is about 0.1 μM. Because the K_m for Mg·ATP is estimated from Fig. 5 to be 70 μM, the inhibitor has about 700 times the affinity of Mg·ATP for the active site.

We have determined that the Al·ATP complex in the absence of Mg is not measurably active by our methods as a substrate for hexokinase. Because solvent exchange data for Al^{3+} com-

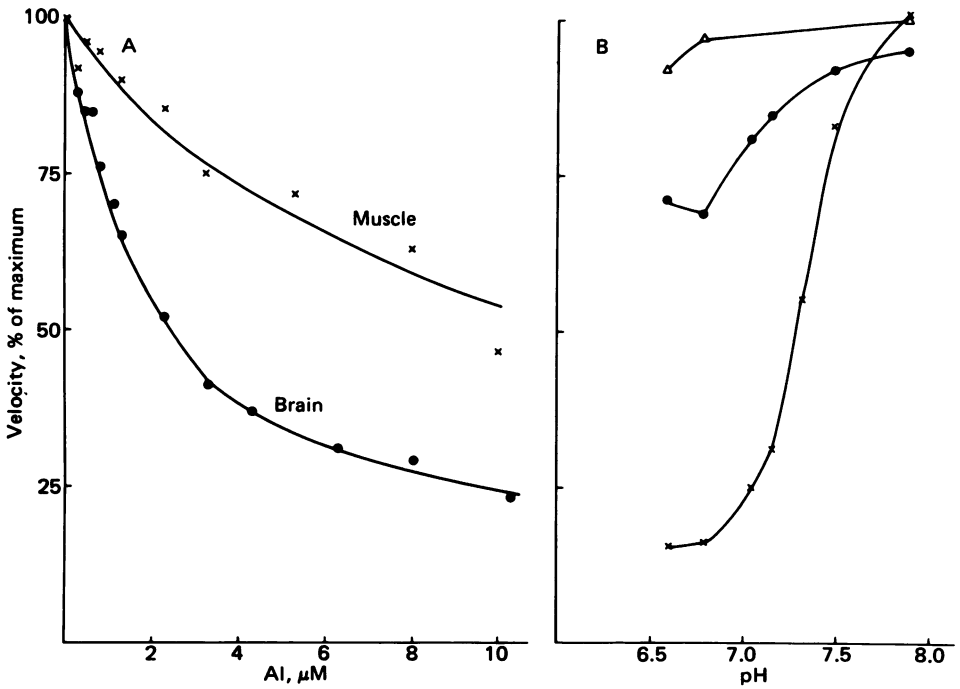

FIG. 6. (*A*) Effect of varying aluminum content of ATP on activity of brain (type I) and muscle (type II) hexokinase. The experiment was analogout to that in Fig. 2*A*. (*B*) Effect of pH on aluminum inhibition of rat brain type I hexokinase. The experiment was analogous to that in Fig. 3, except that the ATP concentration was 0.5 mM. The activity with citrate was the same without or with added aluminum at pH 6.9. Δ, Activity with citrate; O, activity with no added aluminum; X, activity with 5 μM aluminum added.

plexes give rate constants in the range of 0.1–3.0 sec^{-1} (14), compared with rate constants of 100–400 sec^{-1} for the hexokinases, it seems possible that, in the complex of Al·ATP with hexokinase, the rate of phosphate transfer may be limited by the rate at which Al^{3+} coordination bonds with the nucleotide or protein can be broken.

There is a strong dependence of the Al^{3+} inhibition on pH (Figs. 3 and 6). The basis for this pH dependence remains to be determined. It seems likely that the two or three protons involved in the inhibition are used for titration of the Al^{3+} to the effective ionic form, perhaps for optimal formation of the inhibitory Al·ATP complex. In studies based on the present findings, Bock *et al.* (Jay Bock, David Ash, and George Reed, personal communication) have noted profound effects of Al^{3+} on the ^{31}P NMR and infrared spectra of ATP, but only at pH values below 7.

The high sensitivity of the brain enzyme to Al^{3+} suggests a possible connection to the recently discovered role of aluminum in the dialysis encephalopathy syndrome (15). In uremic patients undergoing dialysis there is a high incidence of severe neurological symptoms accompanied by a high accumulation of aluminum in the brain gray matter. There is also evidence for increased brain aluminum levels in patients with senile dementia (Alzheimer disease) (16).

The Al·ATP complex shows striking specificity, inhibiting various hexokinases but not yeast glucokinase or muscle phosphofructokinase. Further work on specificity is needed. In a recent paper (17) it has been reported that the inhibition of liver fructose-bisphosphatase by an ATP preparation is due to the presence of an unidentified contaminating metal. Because the ATP used was lot 51001 from P-L laboratories, which we report here to be heavily contaminated with aluminum, it will be of interest to study the effect of Al^{3+} (or Al·ATP) in that system.

The vanadate present in certain ATP preparations has recently been shown to be responsible for inhibition and anomalous kinetics of Na^+,K^+-ATPases (18). It is clear from the present work that vanadate plays no role in the inhibition of hexokinases at low pH. Conversely, aluminum has very little effect on the Na^+,K^+-ATPase of guinea pig kidney membranes; 20 μM Al^{3+}, added as an Al·ATP complex to a reaction mixture containing 2 mM Mg·ATP at pH 7.0, caused only 16% inhibition (Robert Post, personal communication).

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