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Kinetic Properties of Hexokinase under Near-Physiological Conditions: Relation to Metabolic Arrest in Artemia Embryos During Anoxia

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Kinetic Properties of Hexokinase under Near-physiological Conditions
RELATION TO METABOLIC ARREST IN ARTEMIA EMBRYOS DURING ANOXIA

Bernard B. Rees, Ira J. Ropson, and Steven C. Hand

Previous analyses of glycolytic metabolites in Artemia embryos indicate that an acute inhibition of glucose phosphorylation occurs during pH-mediated metabolic arrest under anoxia. We describe here kinetic features of hexokinase purified from brine shrimp embryos in an attempt to explain the molecular basis for this inhibition. At saturating concentrations of cosubstrate, ADP is an uncompetitive inhibitor toward glucose and a partial noncompetitive inhibitor toward ATP (K_i = 0.86 mM, K_a = 1.0 mM, K_ad = 1.9 mM). With cosubstrates at subsaturating concentrations, the uncompetitive inhibition versus glucose becomes noncompetitive, while inhibition versus ATP remains partial noncompetitive. The partial noncompetitive inhibition of ADP versus ATP is characterized by a hyperbolic intercept replot. These product inhibition patterns are consistent with a random mechanism of enzyme action that follows the preferred order of glucose binding first and glucose-6-P dissociating last. We propose that in vivo, in an attempt to explain the molecular basis for metabolic arrest under anoxia, hexokinase inhibition occurs while cellular levels of glucose-6-P are decreasing, other regulatory features in addition to product inhibition must be operative. In the present study, a molecular and kinetic analysis was undertaken with hexokinase purified from post-diapause Artemia embryos to explain the inhibition of this enzyme that occurs in vivo during the transition between active and quiescence metabolic states.

Recent observations indicate that alterations of intracellular pH (pHi) may play a primary role in the regulation of carbohydrate metabolism in Artemia. pH, declines rapidly from values ≥7.9 during aerobic development to 6.8 during anaerobic incubation, eventually reaching pH 6.3 after several hours (Busa et al., 1982). Artificial acidification of pH, to 6.8 by elevated CO_2 (aerobic acidosis) results in a suppression of respiration rate (Busa and Crowe, 1983) and a blockage of carbohydrate metabolism characterized by cross-over points essentially identical to those under anoxic conditions (Carpenter and Hand, 1986a). It is appropriate to note that while both the ATP:ADP ratio and adenylate energy charge sharply decline under anoxia, they remain constant under aerobic acidosis (Carpenter and Hand, 1986a). Thus, changes in adenylates are not necessary for the observed hexokinase inhibition. As a consequence, all experiments in the present communication, including characterization of hexokinase reaction mechanism and studies of subcellular distribution and inhibition characteristics, were performed at pH 8.0 and 6.8 in order to detect any pH-dependent changes in these properties.

Finally, Womack and Colowick (1979) reported that aluminum ion at micromolar concentrations can inhibit hexokinase activity in a pH-dependent manner. Until now, studies have not experimentally addressed the potential metabolic significance of this finding, but rather have viewed aluminum only as a common contaminant of ATP preparations. Consider...
ering the acute pH transition that occurs during entry into anaerobic dormancy, we felt it germane to measure the alu-
minal content of Artemia embryos and evaluate the inhibi-
tion of hexokinase at physiological levels of this metal ion
and its major cellular chelators.

**EXPERIMENTAL PROCEDURES**

In all cases, concentrations of the varied substrate bracketed the $K_m$ value, and assays were typically performed in duplicate. When values deviated by more than 10%, quadruplicate assays were done. The average standard error within a set of assays was 5.1% of the quadruplicate mean. Lineweaver-Burk plots of initial velocity mea-
surements were constructed for diagnostic purposes, but the lines
given are those predicted by the appropriate rate equations. The
nonlinear least squares regression procedure of the Statistical Analy-
sis System (SAS Institute, Cary, NC) was used to determine kinetic
parameters, their standard errors, and goodness of fit characteristics.
The equations used to fit the various kinetic models were adopted
from Segel (1975).

**RESULTS**

**Physical Properties and Substrate Specificity—**Hexokinase purified from Artemia embryos has a native molecular weight of 40,000 ± 1,500 (S.E., n = 3) based on gel exclusion chroma-
tography with Sephacryl S-200. This $M_r$ is consistent with
reported values from other invertebrates (Stetten and Gold-
The isoelectric point of Artemia hexokinase was determined
by chromatofocusing to be between pH 4.35 and 4.50, which
is consistent with $pI$ values reported for yeast and
mammalian type I hexokinase. In contrast, substitution or isomerization at the C-2 position (2-deoxy-D-glucose, glucosamine; mannoe) does not reduce activity as severely. Di- and trisaccharides were not good sub-
strates. In a separate experiment, enzyme activity was meas-
ured in the presence of 3 mM inosine triphosphate and 5 mM
ATP and was found to be approximately 10% of the activity
with ATP as phosphoryl donor. This level of activity with
ITP is comparable to other hexokinases, but is substantially
higher than that observed for mammalian glucokinase (Col-

**Kinetic Studies with Artemia Hexokinase—**The reaction mechanism for Artemia hexokinase was studied by examining patterns of product inhibition at pH 8.0 and 6.8. Results obtained at pH 8.0 are shown in Figs. 1–4 (Miniprint) and are described below. Since the patterns of inhibition observed at
pH 6.8 were generally similar, these results will only be ad-
ressed when qualitatively distinct.

Plots of $1/velocity$ versus $1/[ATP]$, and $1/velocity$ versus
$1/[glucose]$ at several concentrations of cosubstrate resulted in
intersections in the second quadrant. These results support a
ternary complex reaction mechanism for Artemia hexoki-

nase. There was no indication of substrate inhibition by either
glucose or ATP up to concentrations of 10 and 5 mM, respect-
ively.

At saturating concentrations of cosubstrate, ADP was found to be an uncompetitive inhibitor toward glucose and a
partial noncompetitive inhibitor toward ATP. In experiments
with ATP fixed near its $K_m$, the uncompetitive inhibition
versus glucose becomes noncompetitive. When glucose was
held at subsaturating concentrations, the inhibition versus
ATP remained partial noncompetitive. The partial noncom-
petitive inhibition is characterized by a hyperbolic replot of
intercepts, and is described by Equation 5. At all four com-
binations of pH (8.0 and 6.8) and glucose (5 and 0.2 mM),
fitting the data to this equation reduced the residual sums of
squares compared to the fit provided by the standard descrip-
tion of noncompetitive inhibition (Equation 4). Inhibition by
glucose-6-P was somewhat more complex. The pattern was
noncompetitive versus glucose at pH 6.8, whereas, inhibition
at pH 8.0 was uncompetitive. At high glucose-6-P concentra-
tions (≥4 mM, Fig. 3), the values depart from those predicted by
the uncompetitive model, which suggests a noncompetitive

### Table II

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Velocity $^a$</th>
<th>Sugar</th>
<th>Velocity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM sugar</td>
<td>100 mM sugar</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>91</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>51</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Fruuctose</td>
<td>7</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>2</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Xylose, galactose, arabinose $^b$</td>
<td>≤ 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Rate of ADP formation was measured in the presence of 3 mM
ATP and 24 mM MgCl$_2$ (pH 8.0) and expressed as the percent of
enzyme activity measured with glucose. Values for 100 mM sugar were
determined with partially purified enzyme.

$^b$ Rate of reaction not measured at 100 mM due to confounding
effects of trace glucose contamination in these sugars.
Artemia hexokinase are summarized in Table I. Analysis of ATP was competitive at concentrations of glucose-6-P less than 1 mM (Equation 2). Since the K_i for ATP and the K_i for ADP are near the dissociation constants of ATP and ADP (pK_i, 7.0; pK_i, 6.8), the enzyme activity is reduced by about 62% at conditions found in dormant anaerobic embryos (pH 6.3; adenylate energy charge, 0.81) is only depressed by 28% relative to the aerobic value. Because there is a dramatic inhibition of hexokinase in vivo under aerobic acidosis, we felt that there was likely another effector influencing enzyme activity at low pH.

In addition to glucose-6-P and ADP, several other metabolites were evaluated for potential inhibitory effects of Artemia hexokinase activity. Glucose 1,6-diphosphate is claimed to be a potent inhibitor of hexokinase at low pH (Beitner, 1985); but, at concentrations up to 500 μM, this compound did not alter enzyme activity at pH 8.0, 6.8, or 6.3 in the presence of 1.5 mM ATP and 3 mM MgCl_2. Fructose, 1,6-diphosphate, fructose 2,6-diphosphate, and guanosine diphosphates were also ineffective as inhibitors.

**Inhibition pattern**

- UC: competitive
- NC: noncompetitive
- UC: partial noncompetitive
- C: uncompetitive

**Parameter values**

- Parameter values determined by fitting to the equation for a sequential reaction mechanism (Equation 1).
- Parameter values determined by fitting to the equation for partial noncompetitive inhibition (Equation 5) at saturating glucose.
- Parameter values determined by fitting to the equation for partial noncompetitive inhibition (Equation 4).

### Table IV

**Kinetic constants of Artemia hexokinase**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH 8.0</th>
<th>pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_a glucose</td>
<td>0.16 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>K_a ATP</td>
<td>0.26 ± 0.01</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>K_a glucose</td>
<td>0.82 ± 0.12</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>K_a ADP</td>
<td>0.86 ± 0.09</td>
<td>0.79 ± 0.28</td>
</tr>
<tr>
<td>K_a ADP</td>
<td>1.0 ± 0.3</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>K_a glucose-6-P</td>
<td>0.065 ± 0.005</td>
<td>0.080 ± 0.006</td>
</tr>
<tr>
<td>K_a glucose-6-P</td>
<td>5.4 ± 2.9</td>
<td></td>
</tr>
</tbody>
</table>

**Parameter values**

- Parameter values determined by fitting to the equation for a sequential reaction mechanism (Equation 1).
- Parameter values determined by fitting to the equation for partial noncompetitive inhibition (Equation 5) at saturating glucose.
- Parameter values determined by fitting to the equation for partial noncompetitive inhibition (Equation 4).

### Table V

**Intracellular pH and metabolite concentrations during active and quiescent metabolic conditions in Artemia embryos**

Metabolite concentrations are expressed as mM ± 1 S.E. (n = 3-6).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Aerobic development</th>
<th>Aerobic acidosis</th>
<th>Anaerobic dormancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>≥ 7.9</td>
<td>6.8</td>
<td>6.3</td>
</tr>
<tr>
<td>ATP</td>
<td>1.17 ± 0.06</td>
<td>1.22 ± 0.02</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>ADP</td>
<td>0.35 ± 0.02</td>
<td>0.37 ± 0.02</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>AMP</td>
<td>0.09 ± 0.05</td>
<td>0.16 ± 0.03</td>
<td>1.16 ± 0.20</td>
</tr>
<tr>
<td>Phosphate</td>
<td>12.2 ± 0.3</td>
<td>11.4 ± 0.1</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.88 ± 0.01</td>
<td>0.99 ± 0.03</td>
<td>1.10 ± 0.08</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>0.203 ± 0.002</td>
<td>0.057 ± 0.003</td>
<td>0.056 ± 0.006</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.65 ± 0.04</td>
<td>0.46 ± 0.01</td>
<td>0.46 ± 0.04</td>
</tr>
</tbody>
</table>

### Table III

**Product inhibition of Artemia hexokinase**

<table>
<thead>
<tr>
<th>Varied substrate</th>
<th>Fixed substrate</th>
<th>Varied product</th>
<th>Inhibition pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 5 mM ATP</td>
<td>ADP</td>
<td></td>
<td>UC</td>
</tr>
<tr>
<td>Glucose 0.3 mM ATP</td>
<td>ADP</td>
<td></td>
<td>NC (p &gt; 0.95)</td>
</tr>
<tr>
<td>ATP 5 mM glucose</td>
<td>ADP</td>
<td></td>
<td>pNC (p &gt; 0.999)</td>
</tr>
<tr>
<td>ATP 0.2 mM glucose</td>
<td>ADP</td>
<td></td>
<td>pNC (p &gt; 0.999)</td>
</tr>
<tr>
<td>Glucose 5 mM ATP</td>
<td>Glucose-6-P</td>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>ATP 5 mM glucose</td>
<td>Glucose-6-P</td>
<td></td>
<td>C</td>
</tr>
</tbody>
</table>

* C, competitive; NC, noncompetitive; UC, uncompetitive; pNC, partial noncompetitive (slope-linear, intercept-hyperbolic).

**p values**

- p values represent the probability that the NC model improved the fit over the UC model.
- p values represent the probability that the pNC model improved the fit over the NC model.
Inhibition of hexokinase activity at near-physiological levels of pH, metabolites, aluminum, and aluminum chelators

Values of pH and metabolite concentrations corresponding to the appropriate metabolic condition were as in Table V, except that glucose in all assays was 1.0 mM. Aluminum (as AlCl3) was 72 μM, and MgCl2 was 2 mM. Enzyme rate was measured as the number of micromoles of glucose-6-P formed per min in a 1.0-mL reaction mixture over 25 min at 25 °C. Blanks were reaction mixtures with deionized water replacing enzyme. Incubations were stopped by the addition of perchloric acid to 6%. Following neutralization by potassium carbonate, glucose-6-P was measured fluorometrically (Lowry and Passonneau, 1972). Activity values are means ± 1 S.E. (n = 3).

<table>
<thead>
<tr>
<th>Metabolic condition</th>
<th>Hexokinase activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic development (control)</td>
<td>1.39 ± 0.20</td>
<td>100</td>
</tr>
<tr>
<td>Aerobic acidosis</td>
<td>0.19 ± 0.03</td>
<td>12</td>
</tr>
<tr>
<td>Anaerobic dormancy</td>
<td>0.14 ± 0.06</td>
<td>9</td>
</tr>
</tbody>
</table>

The primary focus of this study was to examine the catalytic and physical properties of hexokinase from Artemia embryos in order to identify the molecular characteristics responsible for the reduction in flux at this step in glycolysis during metabolic arrest in the species (Carpenter and Hand, 1986a). Product inhibition, reaction mechanism, subcellular distribution, and aluminum inhibition are interpreted in the context of metabolic control in Artemia embryos, and related to hexokinases from other sources. The results have implications for the evolution of hexokinase homologues.

**Reaction Mechanism of Artemia Hexokinase**—The patterns of product inhibition observed in the present study are not characteristic of typical ordered or random bireactant mechanisms. However, when all the data are considered, they are consistent with a random mechanism with the preferred order of glucose binding first and glucose-6-P dissociating last. A preference for this route of substrate addition and product release has been reported for hexokinases from yeast (Dunberg and Cleland, 1975) and mammals (Ganson and Fromm, 1985), although the extent to which the mechanism is random is currently debated (Gregorou et al., 1986; Bass and Fromm, 1987). We feel that in Artemia hexokinase, the pathway is effectively ordered at physiological concentrations of substrates and products, but the random mechanism is required to explain some of the inhibition patterns at high levels of products. It is appropriate to note that the equations of Rudolph and Fromm (1971) for a rapid equilibrium random mechanism do not apply to Artemia enzyme. Scheme I sum-

**FIG. 5. Aluminum inhibition of Artemia hexokinase.** A. Assay conditions were 1 mM glucose, 1 mM ATP, 5 mM MgCl2, and pH 8.2 (■) or 7.0 (○). The activity at 0 μM aluminum was measured in the presence of 0.5 mM sodium citrate, B. pH dependence of inhibition by aluminum. Control conditions were 1 mM ATP, 1 mM glucose, 2 mM MgCl2, and 0.5 mM citrate (△). Assays at 72 μM AlCl3 were performed in the absence (□) and presence (○) of physiological metal chelators (0.5 mM sodium citrate and 12.5 mM sodium phosphate). Points represent means of two or three determinations of reaction rate and expressed as the percent of the activity at pH 8.0. Inset, the effect of aluminum concentration on hexokinase activity measured at pH 6.8 in the presence of 0.5 mM citrate and 12.5 mM phosphate.
marizes our interpretation of product inhibition studies with Artemia hexokinase.

Support for the ordered nature of the mechanism is found in the pattern of inhibition by ADP. In the proposed mechanism, ADP is usually the first product released and, as such, is expected to be a noncompetitive inhibitor when either substrate is held at unsaturated concentrations. At saturating concentrations of first substrate (glucose), the predicted pattern of inhibition versus the second substrate (ATP) remains noncompetitive. In contrast, when the second substrate (ATP) is held at saturating concentrations, the pattern of inhibition versus the first substrate (glucose) should change to uncompetitive. Our data fulfill these predictions.

The slope-linear, intercept-hyperbolic noncompetitive inhibition of ADP versus ATP has been previously documented for yeast hexokinase (Kosow and Rose, 1970; Viola et al., 1982). The explanation offered by Kosow and Rose (1970) for this pattern recognizes two sites of ADP inhibition, an effect on product release and a competitive interaction versus ATP at the active site. As inhibitor and substrate concentrations are increased, the interaction becomes predominantly competitive, giving a hyperbolic replot of intercepts. The observation of competitive inhibition of ADP toward ATP suggests that the mechanism is partly random at high concentrations of substrate and product (Viola et al., 1982). Scheme I includes the formation of an enzyme-glucose-ADP dead-end complex to account for the competitive portion of the hyperbolic inhibition.

Competitive inhibition of glucose-6-P versus ATP is not predicted in a typical random mechanism, but can be resolved by the formation of the dead-end complex enzyme-glucose-6-P (Scheme I). In a random mechanism, a competitive inhibitor of ATP would be expected to be noncompetitive versus glucose (Fromm, 1983), and this pattern is observed in a variety of hexokinases (Purich et al., 1973). Fitting our glucose-6-P versus glucose data to a noncompetitive model reduced the residual sums of squares relative to the uncompetitive model at both pH 8.0 and 6.8. The improvement in the fit was not significant at pH 8.6. Thus, the simpler model (uncompetitive) was accepted as suggested by Mannervik (1983). This uncompetitive inhibition supports the proposal that there is a preferred route of substrate addition for Artemia hexokinase, since a competitive inhibitor of the second substrate in an ordered mechanism would be uncompetitive versus the first substrate (Fromm, 1983). At the lower pH, the switch to noncompetitive inhibition may reflect either an increased flux through the alternative route of substrate addition or a secondary interaction of glucose-6-P at the glucose site.

Considerable debate exists about the nature of glucose-6-P inhibition of mammalian hexokinases. Fromm and colleagues (Bass and Fromm, 1987) hold that inhibition results from competition between glucose-6-P and ATP for the γ-phosphate subsite of the active site, while Cornish-Bowden and others (Gregorius et al., 1986) favor allosteric modification by glucose-6-P. The latter position is supported by structural studies that present evidence for gene duplication-fusion in the evolution of mammalian hexokinases (White and Wilson, 1987; Schwab and Wilson, 1988). Briefly, the argument holds that the ancestral hexokinase was of the order of 50,000 Da and duplication of the gene coding for this protein, followed by fusion of the gene copies, has resulted in a 100,000-Da molecule. One active site remained catalytically competent, while an allosteric site arose from modification of the alternative active site. While this may be the case in mammalian isozymes, it cannot adequately explain the acute inhibition by glucose-6-P of Artemia enzyme, which has a native molecular weight of 40,000. Consequently, we feel that the inhibition by glucose-6-P is a result of competition with ATP at the active site.

Absence of Hexokinase Interaction with Mitochondria—Another feature of the enzyme which can be rationalized in light of structural differences between mammalian and Artemia hexokinases is the capacity to bind to mitochondria. Mammalian brain hexokinase possesses three discrete domains: a C-terminal 10,000-Da polypeptide; a middle domain of 50,000 Da; and a 40,000-Da C-terminal segment (White and Wilson, 1987). The N-terminal polypeptide mediates binding to mitochondria, whereas the catalytic site resides within the C-terminal portion (White and Wilson, 1987). The Artemia enzyme is expected to be homologous to the mammalian C-terminal region, thus lacking the peptide responsible for reversible mitochondrial binding.

Influence of Substrates and Metabolites under in Vivo Conditions—The importance of the individual kinetic constants and pH-related alterations thereof in determining enzyme rate in vivo can only be assessed in context of cellular metabolite concentrations. Using an equation for a sequential mechanism that incorporates competitive inhibition by glucose-6-P (Segel, 1975) and kinetic constants from Table IV (and including a 20% reduction in Vₐ at pH 6.8 relative to pH 8.0), we calculated hexokinase activity at physiological substrate and product concentrations (Table V). The calculated rate of hexokinase under anaerobic conditions, applying the pH 6.8 kinetic parameters, is 32% lower than the value calculated for aerobic conditions. Hence, while the decrease in glucose-6-P predicts an activation relative to aerobic conditions, a marked decline in ATP concentration results in moderate inhibition of the enzyme. During the transition from aerobic development to aerobic acidosis, however, ATP levels do not change while glucose-6-P declines, and the predicted velocity under these conditions represents an activation of 20% above aerobic conditions. Because the ADP concentration is relatively stable under these treatments, inhibition by this product should be similar in all three cases. Thus, product inhibition alone cannot explain the negative cross-over observed at the hexokinase reaction during aerobic acidosis (Carpenter and Hand, 1986a), and we felt that another effector is likely to be inhibiting enzyme activity at low pH values.

Aluminum Inhibition: Possible Significance for Metabolic Arrest—Aluminum ion, which has been demonstrated to be a powerful inhibitor of yeast and mammalian hexokinases, is likewise a potent effector of Artemia hexokinase at low pH. This inhibition is presumably due to the formation of Al-ATP which competes with Mg-ATP for the nucleotide binding site (Womack and Colowick, 1979; Neet et al., 1982). Because 50% maximum inhibition occurs at about 0.5 μM aluminum (Fig. 5A) and the Kₘ for Mg-ATP is 290 μM, the Al-ATP appears to bind approximately 3 orders of magnitude tighter to the enzyme than does the substrate. Neet et al. (1982) proposed that Al-ATP binds to the enzyme-glucose complex, inducing a slow conformational change to an enzyme
form which is not catalytically competent. While only steady state velocities are reported for *Artemia* hexokinase, the observation that citrate activation of the inhibited enzyme required 1–2 min prior to establishment of a linear rate supports this suggestion. Although inhibition by aluminum is severe at pH values below neutrality, the effect is not observed above pH 8.0. There is a number of explanations for this lack of inhibition at higher pH values. The effect of increasing solution pH on mononuclear aluminum species causes the free Al\(^{3+}\) concentration to drop dramatically as pH rises (Martin, 1986). High pH values foster the formation of polynuclear aluminum species (Ackett et al., 1972), which may be too large to be complexed with ATP. Finally, Al-ATP\(^{2-}\) has a pK value of 7.6 and is much less effective as a competitor of Mg-ATP\(^{2-}\) above this pH (Viola et al., 1980).

To our knowledge prior studies have not experimentally addressed aluminum inhibition of hexokinase at in vivo values of pH and metal ion chelators. At pH values, metabolite, aluminum, and chelator concentrations corresponding to an aerobic dormancy and aerobic acidosis, hexokinase activity in vitro was inhibited by approximately 90% compared to the activity under conditions which stimulate aerobic development. Our measurements of enzyme activity were performed at 72 μM aluminum, which assumes that the total tissue aluminum exists in soluble form. We recognize that aluminum can exist in small molecules as well as some proteins, and thus we included in our assays its major cellular chelators (citrate and phosphate) at concentrations as some proteins, and thus we included their major cellular chelators (citrate and phosphate) at concentrations that reflect total tissue content. Aluminum inhibition could also have implications for Alzheimer’s disease (Crapper et al., 1976), dialysis encephalopathy syndrome (Alfrey et al., 1972), which may be too large to be complexed with ATP. Finally, Al-ATP\(^{2-}\) has a pK value of 7.6 and is much less effective as a competitor of Mg-ATP\(^{2-}\) above this pH (Viola et al., 1980).

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**Experimental Procedures**

Materials: DEAE-cellulose, Sephadex G-200, Polybuffer 74, Polybuffer exchanger 94, glucose-6-P dehydrogenase (yeast, type VII), pyruvate kinase (rabbit muscle, type II), lactate dehydrogenase (rabbit muscle, type I), NADH, NADPH, and NAPDH were purchased from Sigma Chemical Company. All other chemicals were reagent grade. Buffers and reagents were prepared in concentrated solutions in water: 

- **TABLE I:** Purification of hexokinase from Artemia embryos.

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity (units)</th>
<th>Specific Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg Supernatant</td>
<td>406</td>
<td>0.08</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>214</td>
<td>0.14</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>146</td>
<td>0.75</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>98</td>
<td>1.1</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>57</td>
<td>88</td>
</tr>
</tbody>
</table>

**Hemolymph Analysis:** Hexokinase activity was measured spectrophotometrically (340 nm) at 25°C by following the production of glucose-6-P from glucose-6-P dehydrogenase (type VU), pyruvate kinase (rabbit muscle, type II), lactate dehydrogenase (rabbit muscle, type I), NADH, NADPH, and NADPH were purchased from Sigma Chemical Company. All other chemicals were reagent grade. Buffers and reagents were prepared in concentrated solutions in water.

**Activity measured at rate of glucose-6-P production in the presence of 5 mM glucose, 3 mM ATP and 5 mM MgCl₂, pH 8.0**

**Hexokinase Assay:** Hexokinase activity was measured spectrophotometrically at 340 nm at 25°C by following the production of glucose-6-P at 0.05 mM NADP, 3 mM phosphate buffer at pH 7.5, 3 mM glucose, 3 mM MgCl₂, pH 8.0. The assay buffer was 0.05 mM NADP, 3 mM phosphate buffer at pH 7.5, 3 mM glucose, 3 mM MgCl₂, pH 8.0. The assay was initiated by the addition of the indicator reagent.

**We measured aluminum in Sigma disodium ATP from the endogenous level of ATP (see Methods) at 0.05 mM NADP, 3 mM phosphate buffer at pH 7.5, 3 mM glucose, 3 mM MgCl₂, pH 8.0. The assay was initiated by the addition of the indicator reagent.**

RESULTS

FIGURE 1: Double reciprocal plots of velocity (μmol min⁻¹) versus glucose concentration (mM) at varied concentrations of ADP. Assays were performed at pH 8.0, and ATP was fixed at 5 mM. ADP concentrations: 0 mM (●), 0.5 mM (○), 1.0 mM (△), 2.0 mM (■), 3.0 mM (▲), and 8.0 mM (×). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the non-competing model of inhibition (goodness of fit, $r^2 = 0.991$).

FIGURE 2: Double reciprocal plots of velocity (μmol min⁻¹) versus ATP concentration (mM) at varied concentrations of ADP. Assays were performed at pH 8.0, and glucose was fixed at 5 mM. ADP concentrations were as in Figure 1. Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the slope-linear, intercept-hyperbolic non-competitive model of inhibition ($r^2 = 0.984$).

FIGURE 3: Double reciprocal plots of velocity (μmol min⁻¹) versus glucose concentration (mM) at varied concentrations of glucose-6-P. Assays were performed at pH 8.0, and ATP was fixed at 5 mM. Glucose-6-P concentrations were: 1 mM (●), 1.0 mM (○), 1.1 mM (△), 1.2 mM (■), 1.3 mM (▲), and 1.6 mM (×). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the competitive model of inhibition ($r^2 = 0.985$).

FIGURE 4: Double reciprocal plots of velocity (μmol min⁻¹) versus ATP concentration (mM) at varied concentrations of glucose-6-P. Assays were performed at pH 8.0, and glucose was fixed at 5 mM. Glucose-6-P concentrations were: 0 mM (●), 0.1 mM (○), 0.4 mM (△), and 1.0 mM (×). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the competitive model of inhibition ($r^2 = 0.983$, see Results).