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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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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 (\(K_a = 0.86 \text{ mM}, K_d = 1.0 \text{ mM}, K_{id} = 1.9 \text{ mM}\)). 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 the formation of the dead-end complex, enzyme-glucose-phosphate-6-P. Versus glucose, inhibition by glucose-6-P (\(K_i = 65 \text{ mM}\)) occurs primarily by competing with ATP at the active site, resulting in the 6.8. Over a physiologically relevant pH range of 8.0 to 6.8 alterations in \(K_a\) and \(K'_a\) values do not account for the reduction in glucose phosphorylation, and no evidence suggests that Artemia hexokinase activity is modulated by reversible binding to intracellular structures. Total aluminum in the embryos is \(4.01 \pm 0.36 \mu g/dry\ weight\), or, based upon tissue hydration, 72 \mu m. This concentration of aluminum dramatically reduces enzyme activity at pH values < 7.2, even in the presence of physiological metal ion chelators (citrate, phosphate). When pH, aluminum, citrate, phosphate, substrates, and products were maintained at cellular levels measured under anoxia, we can account for a 90% inhibition of hexokinase relative to activity under control (aerobic) conditions.

Hexokinase (EC 2.7.1.1) plays a major role in the regulation of carbohydrate metabolism in mammalian cells that utilize glucose as a primary energy source. Glucose-6-phosphate, a product and potent inhibitor of the hexokinase reaction, is presumed to be the primary regulator of activity (Colowick, 1973), although alterations in the subcellular distribution of the enzyme are physiologically important depending on the tissue (Wilson, 1980; 1985). It is thought that a negative cross-over point in metabolite concentrations, indicative of enzyme inhibition, is typically not observed at the hexokinase reaction because of product inhibition by glucose-6-P (Rollston, 1972). However, this scenario is clearly not applicable to embryos of the brine shrimp, Artemia. Biochemical and calorimetric studies have shown that the predominantly trehalase-based metabolism of the developing embryo is brought to a virtual halt upon anaerobic incubation and is quickly reinitiated upon return to aerobic conditions (Ewing and Oegg, 1969; Carpenter and Hand, 1986a; Hand and Gnaiger, 1988). Analyses of glycolytic intermediates in these embryos during aerobic development and anaerobic dormancy have demonstrated that trehalase, hexokinase and phosphofructokinase are inhibited under anaerobic conditions (Carpenter and Hand 1986a, 1986b; Hand and Carpenter, 1986). Because the 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 extracellular phosphorus (pH) may play a primary role in the regulation of carbohydrate metabolism in Artemia. pH, declines rapidly from values \(\geq 7.9\) 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} (acidicosis) 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 characterics, 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 ions 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 aluminim content of Artemia embryos and evaluate the inhibition 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 measurements 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 Analysis 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 adapted from Segel (1975). Equation 1 predicts enzyme velocity in a sequential mechanism.

$$v = \frac{V_m[A][B]}{K_{A}K_{AB} + K_{A}A + K_{B}B + AB}$$

(1)

Competitive, uncompetitive, and noncompetitive product inhibition were fit to Equations 2, 3, and 4, respectively.

$$v = \frac{V_m[A]}{K_A (1 + I/K_A) + [A]}$$

(2)

$$v = \frac{V_m[A]}{K_{A} + [A] (1 + I/K_N)}$$

(3)

$$v = \frac{V_m[A]}{K_A (1 + I/K_{AB}) + [A] (1 + I/K_B)}$$

(4)

Equation 5 describes partially noncompetitive inhibition.

$$v = \frac{V_m[A]}{K_{A} (1 + I/K_{AB}) + [A] (1 + I/K_N)}/(1 + I/K_N)$$

(5)

In these equations, $v$ is measured velocity, $V_m$ is maximal velocity, $K_A$ and $K_B$ are Michaelis constants, $K_{AB}$ is an apparent dissociation constant, $K_{A}$ is a slope inhibition constant, and $K_N$ and $K_B$ are intercept inhibition constants.

The criteria suggested by Mannervik (1983) and Motulsky and Ransnas (1987) were used to determine which models best fit the data. The univariate statistics procedure of the Statistical Analysis System was used to determine the error structure of the data and to test the normality of the residual distribution. There was no significant relationship between variance and any dependent or independent variable. Thus equal weight was assigned to each measurement for the fitting process.

**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 chromatography with Sephacryl S-200. This $M_r$ is consistent with reported values from other invertebrates (Stetten and Goldsmith, 1981; Moser et al., 1988; Mochizuki and Hori, 1977). The isoelectric point of Artemia hexokinase was determined by chromatofocusing to be between pH 4.35 and 4.50, which is somewhat lower than pH values reported for yeast and mammalian hexokinases (Colowick, 1973; Wilson, 1988).

Artemia enzyme displays substantial catalytic capacity with a variety of sugars (Table II), one characteristic which distinguishes it as a hexokinase rather than a glucokinase. The minimal activity with galactose suggests that an axial orientation of the C-4 hydroxyl group may sterically hinder phosphorylation at the nearby C-6 position, as suggested by Sols and Crane (1954) for mammalian type 1 hexokinase. In contrast, substitution or isomerization at the C-2 position (2-deoxy-D-glucose, glucosamine; mannaose) does not reduce activity as severely. Di- and trisaccharides were not good substrates. In a separate experiment, enzyme activity was measured in the presence of 3 mM inosine triphosphate and 5 mM glucose 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 (Colowick, 1973).

**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 addressed 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 hexokinase. There was no indication of substrate inhibition by either glucose or ATP up to concentrations of 10 and 5 mM, respectively.

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 noncompetitive inhibition is characterized by a hyperbolic replot of intercepts, and is described by Equation 5. At all four combinations 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 description 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 concentrations (≥4 mM, Fig. 3), the values depart from those predicted by the uncompetitive model, which suggests a noncompetitive

| Sugar       | Velocity<sup>a</sup> |
|-------------|---------------------|
|             | 5 mM sugar | 100 mM sugar |
| Glucose     | 100        | 100         |
| 2-Deoxy-D-glucose | 91       | 99          |
| Mannose     | 51         | 77          |
| Glucosamine| 16         | 16          |
| Fructose    | 7          | 98          |
| N-Acetylglucosamine | 2   | 27        |
| Xylose, galactose, arabino<sup>b</sup> | ≤ 1 |

<sup>a</sup> Rate of ADP formation was measured in the presence of 3 mM ATP and 24 mM MgCl<sub>2</sub> (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.

<sup>b</sup> Rate of reaction not measured at 100 mM due to confounding effects of trace glucose contamination in these sugars.
Kinetics of Artemia Hexokinase

**TABLE III**

| Varied substrate | Fixed substrate | Varied product | Inhibition pattern \(\text{pH} 8.0\) | Inhibition pattern \(\text{pH} 6.8\) |
|------------------|-----------------|----------------|-----------------|-----------------|
| Glucose          | 5 mM ATP        | ADP            | UC^a             | UC^b            |
| Glucose          | 0.3 mM ATP      | ADP            | NC (\(p > 0.95\))^c | NC (\(p > 0.95\))^c |
| ATP              | 5 mM glucose    | ADP            | pNC (\(p > 0.999\))^c | pNC (\(p > 0.999\))^c |
| ATP              | 0.2 mM glucose  | ADP            | NC              | NC              |
| Glucose          | 5 mM ATP        | Glucose-6-P    | UC^c             | C^b            |
| ATP              | 5 mM glucose    | Glucose-6-P    | NC (\(p > 0.95\))^c | C^b            |

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

^b NC model did not significantly improve the fit.

^c \(p\) values represent the probability that the NC model improved the fit over the UC model.

^d \(p\) values represent the probability that the pNC model improved the fit over the NC model.

**TABLE IV**

Kinetic constants of Artemia hexokinase

| Parameter | \(\text{pH} 8.0\) | \(\text{pH} 6.8\) |
|-----------|-----------------|-----------------|
| \(K_a\), glucose | 0.16 ± 0.01 | 0.12 ± 0.01 |
| \(K_a\), ATP | 0.26 ± 0.01 | 0.29 ± 0.02 |
| \(K_a\), glucose | 0.82 ± 0.12 | 0.20 ± 0.05 |
| \(K_a\), ADP | 0.86 ± 0.09 | 0.79 ± 0.28 |
| \(K_a\), ADP | 1.0 ± 0.3 | 2.6 ± 0.8 |
| \(K_a\), glucose-6-P | 1.9 ± 0.7 | 6.0 ± 3.1 |
| \(K_a\), glucose-6-P | 0.065 ± 0.005 | 0.080 ± 0.006 |

^a Parameter values determined by fitting to the equation for a sequential reaction mechanism (Equation 1).

^b Parameter values determined by fitting to the equation for partial noncompetitive inhibition (Equation 5) at saturating glucose.

^c Value determined by fitting of competitive inhibition of ATP by glucose-6-P at saturating concentrations of glucose and glucose-6-P < 1.0 mM (Equation 2).

^d Value determined by fitting of noncompetitive inhibition of glucose by glucose-6-P when ATP is saturating (Equation 4).

**TABLE V**

Intracellular \(\text{pH}\) and metabolite concentrations during active and quiescent metabolic conditions in Artemia embryos

| Metabolite | Aerobic development | Aerobic acidosis | Anaerobic dormancy |
|-----------|---------------------|------------------|--------------------|
| pH        | \(\geq 7.3\)        | 6.3              | 6.3                |
| ATP       | 1.17 ± 0.06         | 1.27 ± 0.02      | 0.34 ± 0.01        |
| ADP       | 0.35 ± 0.02         | 0.37 ± 0.02      | 0.41 ± 0.01        |
| AMP       | 0.09 ± 0.05         | 0.16 ± 0.03      | 0.16 ± 0.20        |
| Phosphate | 12.2 ± 0.3          | 11.4 ± 0.1       | 15.0 ± 0.3         |
| Glucose^2 | 0.88 ± 0.01         | 0.99 ± 0.03      | 1.10 ± 0.08        |
| Glucose-6-P | 0.203 ± 0.002   | 0.051 ± 0.003   | 0.056 ± 0.006      |
| Citrate   | 0.65 ± 0.04         | 0.46 ± 0.01      | 0.46 ± 0.04        |

^a Intracellular \(\text{pH}\) measured by \(\text{^31P} NMR\) (Busa et al., 1982; Busa and Crowe, 1983).

^b Values taken from Carpenter and Hand (1986a) after 4 h of aerobic development, and after 4 h of aerobic development followed by 4 h of aerobic acidosis or 4 h of anaerobic dormancy.

component at very high levels of inhibitor. Inhibition versus ATP was competitive at concentrations of glucose-6-P less than 1.0 mM, with the inhibition pattern again becoming mixed at higher concentrations. However, the noncompetitive component was seen only at glucose-6-P concentrations 5-20-fold greater than in vivo levels. These data suggest that glucose-6-P inhibits enzyme activity primarily by operating at the ATP site, and only has low affinity for the glucose binding site. The patterns of product inhibition observed for Artemia hexokinase are summarized in Table III. Analysis of slope and intercept replots, with their associated 95% confidence interval, indicated that a linear model adequately describes all data sets except for the intercept-hyperbolic pattern of ADP inhibition versus ATP. The kinetic constants which resulted from the various fits are found in Table IV.

Since the \(K_a\) for ATP and the \(K_a\) for ADP are near the cellular concentrations of these two metabolites, perturbation of the embryonic adenosyl energy charge (Atkinson, 1977) would be expected to influence hexokinase activity. Adenine nucleotides were measured in embryos under conditions of aerobic development, aerobic acidosis, and anaerobic dormancy (Table V). Enzyme activity was assayed at these adenylate levels in the presence of 1 mM glucose and 2 mM MgCl\(_2\) and at applicable pH values. Relative to hexokinase activity at pH 8.0 and energy charge of active embryos (0.83), the enzyme activity is reduced by about 62% at conditions found in dormant anaerobic embryos (pH 6.3; adenosyl energy charge, 0.29). Hence, the combination of lowered pH and energy charge encountered during anaerobic dormancy can account for a significant reduction of hexokinase activity. In contrast, enzyme activity under conditions of aerobic acidosis (pH 6.8; adenosyl 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 \(\mu\)M, 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 diphosphate were also ineffective as inhibitors.

**Aluminum Inhibition**—Aluminum dramatically inhibited Artemia hexokinase activity at pH 7.0 but not at pH 8.2 (Fig. 5A). From these data, half-maximal inhibition at pH 7.0 can be estimated to occur at about 0.5 \(\mu\)M aluminum. Until now, this inhibition has been viewed as an artifact of ATP contamination and biologically unimportant. However, the pH dependence and the severity of the inhibition by aluminum prompted us to investigate the physiological significance of the phenomenon in Artemia embryos.

Total aluminum in Artemia embryos was measured to be 4.0 ± 0.36 \(\mu\)g/g dry tissue (S.E., \(n = 11\)) by graphite furnace atomic absorption spectrophotometry. Based on tissue hydration values, the concentration of aluminum in the embryo is approximately 72 \(\mu\)M. Fig. 5B compares the pH profiles of hexokinase activity in the presence of 72 \(\mu\)M aluminum and in its absence (i.e. 0.5 mM citrate). At a pH value correspond-
Kinetics of Artemia Hexokinase

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-mI 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).

| Metabolic condition       | Hexokinase activity | Relative activity |
|---------------------------|---------------------|-------------------|
|                           | milliunits/ml       | %                 |
| Aerobic development (control) | 1.39 ± 0.20         | 100               |
| Aerobic acidosis          | 0.19 ± 0.03         | 12                |
| Anaerobic dormancy         | 0.14 ± 0.06         | 9                 |

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.

Discussion

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 bimolecular 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 (Dananberg 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-

of aluminum, which reflects the total metal content of the embryo, is presumably reduced in vivo by complexation of Al³⁺ by physiological metal ion chelators. Citrate and orthophosphate are the major small-molecule chelators of aluminum in the cell (Martin, 1986), and their concentrations were also measured in embryos (Table V). When included in assay mixtures, both citrate and phosphate partially offset the aluminum inhibition. However, the pH sensitivity of hexokinase activity is still very acute (circles, Fig. 5B). As shown in the inset to Fig. 5B, this inhibition is observed at pH 6.8 at aluminum concentrations as low as 10 μM.

In a final experiment, we measured hexokinase activity with the discontinuous assay described in Table VI, which permits including both ADP and glucose-6-P simultaneously. The physiological levels of pH, chelators, and metabolites that were used are given in Table V. MgCl₂ was present at 3 mM and AlCl₃ at 72 μM. When compared to the activity under conditions simulating aerobic development, hexokinase is inhibited by 88% under aerobic acidosis and 91% under anaerobic dormancy (Table VI).

Binding Experiments—Hexokinase activity in crude extracts of Artemia embryos is predominantly in the soluble fraction following centrifugation at 20,000 × g. Incubation of 1000 × g supernatants and pellets with 1 mM glucose-6-P (known to elute type I hexokinase from mitochondria; Choa and Wilson, 1972) did not increase hexokinase activity in the 20,000 × g supernatant. In experiments utilizing sucrose homogenization, the soluble hexokinase still accounted for ≥90% of the total enzyme activity. This figure does not change as a function of the metabolic state of the embryos (aerobic development or anaerobic dormancy). Experiments with purified enzyme failed to indicate any interaction of the enzyme with isolated mitochondria or yolk platelets. Thus, we have no evidence at present that regulation of Artemia hexokinase activity is dependent on reversible binding to intracellular structures.
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-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 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 substrate of the active site, while Cornish-Bowden and others (Gregoriou 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 isoforms, 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: an N-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 V, 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 the 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 AI-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 Kₐ for Mg-ATP" is 290 μM, the AI-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

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**Scheme I.** GLC, glucose; G6P, glucose 6-phosphate.
Kinetik of Artemia Hexokinase

15415

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. There are 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 Al³⁺ concentration to drop dramatically as pH rises (Martin, 1986). High pH values favor the formation of polyvalent aluminum species (Akkiet et al., 1972), which may be too large to complex with ATP. Finally Al-ATP− has a pK value of 7.6 and is much less effective as a competitor of Mg-ATP− 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 form complexes with a variety of small molecules as well as some proteins, and thus we included in our assays its 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., 1976), and Parkinsonism (Garruto et al., 1984), all of which are correlated with elevated aluminum concentrations in brain tissue. In this context, Lai and Blass (1984) documented that micromolar amounts of aluminum inhibit glycolysis in mammalian brain extracts.

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2 In the case of citrate, if the intracellular localization is presumed to be largely mitochondrial, then the cytosolic concentration can be calculated to be much lower than the total tissue content. Assuming a 15-fold citrate concentration gradient between mitochondria and cytosol (Williamson, 1979) and a mitochondrial volume of 5% of the total cellular volume (based upon electron micrographs of Artemia embryos), the cytosolic concentration of citrate is calculated to be 40% lower than the total tissue values. At 72 µM aluminum and pH 6.5, decreasing the citrate concentration by this percentage in the hexokinase assay resulted in a similar percent reduction in enzymatic rate. Similarly, a fraction of the measured phosphate is likely sequestered as insoluble calcium salts. By including total tissue levels of aluminum, citrate, and phosphate, we feel that inhibition that would result from overestimated soluble aluminum is offset to some degree by excessive chelator concentration. Ultimately, the degree of aluminum inhibition of hexokinase depends on measured values of free metal ion in the cytosol, which are presently unavailable.
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EXPERIMENTAL PROCEDURES

Materials. DEAE-cellulose, Sephadex G-200, Polybuffer 74, Polybuffer exchanger 94, glucose-6-P dehydratase (yeast, type VII), pyruvate kinase (rabbit muscle, type II), lactate dehydrogenase (rabbit muscle, type I), NAD and NADP were purchased from Sigma Chemical Company. All other chemicals were reagent grade. Buffers and enzymes were made with water distilled by Milli-Q reaggregate system (Millenium Water Systems, Inc.). Enzyme extracts from the brine shrimp were purchased from Sanders Bros. shrimp, Co., Galena, Utah and stored at -20°C.

Enzyme Purification. Unless otherwise specified, all steps were performed at 4°C. After homogenization of eggs (Hud et al., 1983), cytos were homogenized by 3 volumes of homogenization medium (50 mM Tris-HCl buffer, pH 8.0, containing 2 mM DTT, 0.1 mM PMSF, and 1 mg/ml soybean trypsin inhibitor) with a Virtis homogenizer. The instrument was washed with a Mono Ultra Speed rotor and operated at full speed in 4°C for 1 min. Hepernates were filtered through glass wool and centrifuged at 10000 x g to remove ruptured cells and cellular debris. The resulting supernatant was centrifuged at 20000 x g for 30 min.

The enzyme supernatant was fractionated with solid (NH)2SO4, and the protein precipitating between 40 and 60% saturation, was collected by suction filtration at 15000 x g for 1 h. The pellet was resuspended in 50 mM Na-phosphate buffer, pH 7.0, with 1 mM DTT and 0.1 mM PMSF, deayed overnight, and clarified by centrifugation at 15000 x g for 1 h. Washed DEAE-cellulose resin was equilibrated with DEAE buffer and added to the homogenate preincubated at 37°C with 10 mM EDTA. After incubation for 1 h, the resin was washed with DiO buffer and the enzyme buffer containing 100 mM KCl (1.0 million fold ratio).

Hexokinase activity was measured in 300 mM KC1 in DEAE buffer. The enzyme was combined with (NH)2SO4 precipitate, resuspended in a minimal volume of Polybuffer exchanger 150 mM Tris-HCl buffer, pH 7.5, 100 mM glucose, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, and 100 mM KCl, and dialyzed overnight. Aliquots of 2.2 ml were chromatographed on a Sephadex S-200 column (1.5 x 80 cm) at a flow rate of 5.0 ml/min. Hexokinase activity was pooled from all runs. For determination of molecular weight, this gel filtration column was calibrated with the following reference proteins: thyroglobulin, 669,000 (for determination of exclusion volume), catalase, 232,000, aldolase, 159,000; transferrin, 74,000; ovalbumin, 41,000.

The final purification step was chromatography on Polybuffer exchanger 94 (10 x 20 cm column). 23 ml of Polybuffer 74 was used as wash buffer, 25 mM Na-lactate/Tris buffer, pH 6.0, and the elution buffer was 1 M lactate/Tris buffer augmented to pH 4.0 with HCl. Glucose (100 mM) was added to both buffers to help kinase activity in low pH. The enzyme was eluted progressively against the starting buffer following application. Hexokinase activity eluted at pH 4.6 and 5.2, and the enzyme was immediately treated with NaOH and dialyzed against stabilizing buffer (35 mM Tris/HCl, pH 8.0, 2 mM DTT, 50 mM glucose). Throughout the purification, protein was measured by the method of Lowry et al. (1951) as modified by Peterson (1977).

This scheme resolved in a 1500-fold enrichment of hexokinase and gave a 9% recovery of the starting enzyme unit (Table 2). Enzyme purity was 95% determined by the electrophoresis on non-denaturing gels of 1% acrylamide (Davis, 1968). Gels were stained for total protein with fast green dye (Goryachov et al., 1976) and densitometric scanned at 650 nm.

Supplemental Material to

Kinetic properties of hexokinase under near physiological conditions: Calculation of Michaelis Menten constants in Artemia embryos during ammna.

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We measured aluminum in Sigma diatomaceous APT from rice meal (Lee et al., 1980) to be 500 ± 2 ppm (SEM, n = 3), which corresponds to a total ratio of 1:500 (AIP/Al). Attempts to reduce the endogenic aluminum by loss exchange chromatography (Ch pete et al., 1980) lowered the value to 30±70 ppb, comparable with the results of Neet et al. (1982). Because complete removal was not possible with this method, we instead included 0.15 mM sodium citrate in assay buffers to chelate aluminum. To substantiate that citrate addition at low pH values was exclusively a result of aluminum chelation, data were obtained at pH 7.0 and presented in Fig. 5A, which were graphed as a Dixon plot (Segal, 1976). The inositol phosphate at 0.5 mM Al2+ was within 1% of value obtained in the presence of 0.5 mM citrate. In experiments investigating aluminum inhibition, citrate was included only as indicated. In these studies, aluminum (as AICl3), ATP, MgCl2 were preincubated with or without chelatogens in concentrated solutions of ice for 1-h prior to initiation of enzyme assay.

Vindaloo Sauces. The subspecies distribution of hexokinase was evaluated in microbiologically active and quiescent embryos. Following incubation of embryos under conditions of aerobic development or anaerobic dormancy (Cappeletti and Hand, 1986), representative samples of embryos were homogenized in 800 mM sucrose, 1 mM DTT, 0.1 mM PMSF and centrifuged at 20,000 x g for 10 min. A procedure which is known to preserve enzyme activity is evaluated in mammalian skeletal muscle (Clark et al., 1982) and selected invertebrate tissues (Pace and Stoevy, 1966). The supernatants were combined with 3 volumes of solidification buffer (100 mM K-phosphate, pH 7.5, 200 mM KC1, 1 mM EDTA, 0.1 mM PMSF) and assayed for hexokinase activity. The pellets were washed twice in solidification buffer to deleting any bound enzyme.

Binding of purified hexokinase to isolated embryo mitochondria (13 mg/mg protein) was assessed following the protocol of Adams et al. (1983). The mortal interaction of hexokinase and Artemia yolks plants was investigated. Yolk plants were purified as described (Hand and Hand, 1987) and incubated in a concentration of 100 ppb for 20 min with enriched embryos at either 5.0 ± 0.3 (5000 Tmm, 50 µmol buffer with 0.8 µmol) MgCl2). Following centrifugation (170 g), hexokinase activity was measured in supernatant and pellet fractions.

Measurement of metabolites and aluminum. Precocious extracts of isolated Yolk embryos were prepared as in Cappeletti and Hand (1986). Anaerobic nucleotides were measured with high performance liquid chromatography using a procedure modified from Schwartzman and Lee (1980). Nucleotides were separated on an Octadecil column (4.6 x 25 cm; 5 µm particle size) from Jenson Chromatography and stirred isocratically with 0.1 M potassium phosphate buffer, pH 6.0. This HPLC method is superior to enzymatically-coupled fluorometric analyses of APT because it avoids interference by GDP. Cytosine content was determined fluorometrically following the method of Lowry and Passendorf (1977). The 1 ml reaction mixture contained 10 µM NADH, 5 µM ZSnS204, 1 µM exate dehydrogenase and 0.5 µmol 6-phosphate unit eukaryotic hexokinase (buffer pH 7.5). Inorganic phosphate was determined spectrophotometrically as described by Pope and Lwend (1981), except that 7.5% rather than 2.5% ammonium molybdate was included in the reagent.

Aluminum was measured in APT stocks and Artemia embryos following a protocol modified from Cooper et al. (1978). Hypoxygen embryos were treated with an inhibition solution (18.4 mM MgCl2, 60 mM MgCl2, 2.5% active hypothermia) for 30 min on ice to completely remove the oxygen, followed by extensive mixing with denatured H2O. Approximately 100 mg of embryos were transferred to plastic containers, filled with 70°C constant dry weight, and aired at 20°C for 20 h. The ads was raised to 400 µM HNO3 and brought to 1.0 ppm with denatured H2O. Proteins were assayed by conifugation at 100000 x g for 10 min. Al3+ was measured in the supernatant with a Varian Spectrophotometer Absorbent Spectrophotometric.

| Step | Activity (activity units) | Specific Activity (activity units/µg protein) |
|------|------------------------|------------------------------------------|
| 30000rpm Supernatant | 400 | 0.8 |
| Acetaminium Buffer | 216 | 0.14 |
| DEAE-cellulose | 146 | 0.75 |
| Sephacel DE-200 | 91 | 1.1 |
| Chromatography | 59 | 88 |

*Activity measured as rate of glucose-6-P production in the presence of 5 mM glucose, 5 mM ATP and 6 mM MgCl2 (pH 8.0).

Hexokinase Assay: Hexokinase activity was measured spectrophotometrically (430 nm) at 30°C by following the production of glucose-6-P (50 µM NADP; 3.5 mM phosphoenolpyruvate, 10 mM KCl, 0.2 mM MgCl2, 10 mM N-acetylglutamate kinase and lactate dehydrogenase) in Tris-maleate buffer (50 mM). The absorbance of this reaction was measured at 340 nm and the concentration of MgCl2 was adjusted in each reaction mixture so that the concentration of free MgCl2 was between 1 and 3 mM. For the purpose of calculating Mg2+/Cl levels, the stability constants of 20,000, 100, and 1000 were adopted for MgATP, MgADP, (O'Sullivan and Perini, 1986) and MgAMP (Smith and Albeny, 1956). Inhibitors of magnesium was defined as the amount causing the phosphorylation of 1 pmole of glucose per minute.
**RESULTS**

**FIGURE 1:** Double reciprocal plots of velocity (10^2 units) 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 were: 0 mM (O), 0.5 mM (X), 2.0 mM (O), 4.0 mM (A), and 8.0 mM (o). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the uncompetitive model of inhibition (goodness of fit, r² = 0.991).

**FIGURE 2:** Double reciprocal plots of velocity (10^2 units) 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 (r² = 0.984).

**FIGURE 3:** Double reciprocal plots of velocity (10^2 units) 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: 0 mM (O), 0.1 mM (X), 0.4 mM (O), and 1.0 mM (a). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the uncompetitive model of inhibition (r² = 0.991).

**FIGURE 4:** Double reciprocal plots of velocity (10^2 units) 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 (O), 0.1 mM (X), 0.4 mM (O), and 1.0 mM (a). Points represent means of 2-4 determinations of enzyme activity. Lines are predicted by non-linear regression using the competitive model of inhibition (r² = 0.983, see Results).