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REGULATORY PROPERTIES OF LYSINE-SENSITIVE ASPARTOKINASE UNDER EQUILIBRIUM CONDITIONS

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The regulatory properties of the lysine-sensitive aspartokinase (ATP : L-aspartate 4-phosphotransferase, EC 2.7.2.4) have been studied under equilibrium conditions by determining the effects of modifiers on the rate of equilibrium isotope exchange between ADP and ATP. The extent of inhibition by lysine, leucine or phenylalanine is almost independent of substrate concentration but is influenced by the substrate/product ratio. Inhibition by a given concentration of inhibitor is increased when the ADP/ATP ratio is increased indicating a regulatory interaction between end products and cellular energy metabolism. Lysine inhibition is cooperative under equilibrium conditions and the parameters of the Hill equation are nearly identical to those obtained in initial velocity studies. A cooperative heterotropic interaction between lysine and leusine is also observed by the ATP-ADP exchange assay just as it is in initial velocity assays. Thus, the regulatory features of aspartokinase that are observed in initial velocity studies are also manifest under equilibrium conditions as revealed by equilibrium isotope exchange rates.

Introduction

Kinetic and regulatory properties of enzymes are classically studied by initial steady-state velocity measurements. The kinetic and regulatory properties of aspartokinase (ATP : L-aspartate 4-phosphotransferase, EC 2.7.2.4) have been well characterized by this approach [1–5]. That the conditions of such experiments are far from physiological is readily apparent. Substrate concentrations are high, enzyme concentrations are low and products are absent, just the converse of in vivo circumstances [6,7]. While reactions in the cell are not at equilibrium, the mass action ratio of products to substrates is in many cases close to the equilibrium ratio and in all cases there are surely finite concentrations of both substrates and products. Isotope exchange rates therefore provide an opportunity to study flux rates in the presence of product and low substrate, and product concentrations can be used without sacrificing precision. This study was initiated to determine if the regulatory properties of aspartokinase revealed by equilibrium ATP-ADP exchange rates are like those revealed by initial velocity techniques.

Methods

Enzyme preparation. Aspartokinase was purified from *Escherichia coli* B, grown on glucose-salts containing 10 mM leucine, by previously published procedures [4,8]. Aspartokinase was stored at −20°C in 20 mM potassium phosphate buffer (pH 6.75)/0.5 lysine/30 mM β-mercaptoethanol to which 2 vol. of 100% glycerol were added. Aspartokinase was recovered for experiments by dialysis, concentrated by precipitation with $(NH_4)_2SO_4$ (60% saturation) and dissolved in a minimal quantity of the above buffer. Suitable dilutions were made in the buffer without lysine. The resulting concentrations of lysine in the assays were always diluted to a non-inhibitory concentration.

ADP-ATP exchange assay. The lack of ready availability of the unstable β-aspartyl phosphate places some constraints on these equilibrium isotope exchange experiments that are not encountered in cases where all substrates and products are available and stable. Equilibrium must be established enzymatically in each experiment. This requires an incubation of the enzyme with substrates for a period of time sufficient to establish equilibrium prior to the addition of the tracer. The time required to establish equilibrium was determined for each experiment by adding tracer, initially with the substrate, and allowing the reaction to proceed until the distribution of tracer between ATP and ADP became constant. This time was taken as the minimum time required to establish chemical equilibrium. Accordingly, for rate measurements, enzyme and substrates were incubated for this time to establish equilibrium. Then inhibitors (lysine, leucine or phenylalanine) were added where required and the exchange reactions initiated with the addition (40 000 cpm, 0.5 nmol) of [^{14}C]ADP.

Reactions were routinely carried out in a volume of 35 μl and terminated by the addition of 14 μl 25 mM lysine/250 mM EDTA. 10-μl aliquots were then placed in a spot on polyethyleneimine-impregnated (PEI) cellulose thin layer plates (Polygram® Cel 300 by Macherey-Nagel Co.) and developed in 2 vol. 4 M LiCl/8 vol. 1 M acetic acid. Carrier ADP was also added to the spot to facilitate visualization. ATP and ADP were visualized under ultraviolet light, the spots cut out and placed in scintillation vials. 1 ml 100 vol. of 0.7 M $MgCl_2$/1 vol. 2 M Tris-HCl (pH 7.4), was added and the vials were incubated overnight at room temperature (25°C) [9]. Then the ^{14}C content was determined to 2% error by liquid scintillation spectrometry using Triton-X-100/toluene counting-fluid [10]. All asssays were done at pH 8, with 150 mM KCl and excess Mg^{2+} (0.86 mM greater than the total nucleotide concentration). Exchange rates were calculated as described by Boyer [11] using the equation:

$$v = -\frac{[ATP][ADP]}{[ATP] + [ADP]} \frac{1}{t} \ln(1 - F)$$

In this calculation, ATP and ADP concentrations are equilibrium concentrations (μM), t is time (min) and F is fractional attainment of isotopic equilibrium.

The ADP and ATP concentrations were calculated from the initial concentration of ATP and the ratio of ^{14}C between ATP and ADP at equilibrium. A time was chosen such that the value of F would be around 0.5 in the control assay. The reactions were incubated at 25°C in a constant-temperature water bath. Rates were linear with enzyme concentration and $-\ln(1-F)$ was linear with time. ATP, ADP, amino acids and other biochemicals were obtained from Sigma Chemical Company and [8-^{14}C]ADP from New England Nuclear Corporation. PEI thin layer plates were purchased from Brinkman Instruments. Inc., Westbury, NY. Other specific conditions are shown in the figure legends.

Since the equilibrium for this reaction is strongly in favor of ATP and aspartate, traces of ADP in commercial preparations of ATP significantly influence the equilibrium concentration of β-aspartyl phosphate. Therefore, a 9 : 1 ratio of ATP to ADP at equilibrium does not imply a 9 : 1 ratio of aspartate to β-aspartyl phosphate. β-Aspartyl phosphate concentrations are consistent with a mass action ratio of about $5 \cdot 10^{-3}$ [12].

Results and Discussion

Lysine, leucine and phenylalanine are noncompetitive inhibitors of aspartokinase in initial velocity experiments [1,2]. The cooperativity of lysine inhibition of ATP-ADP exchange is demonstrated by the Hill plots in Fig. 1. The parameters of the Hill equation are not significantly affected by substrate concentration and the presence of leucine decreases the cooperativity of lysine inhibition, shifting the curve to lower lysine concentrations. Similar behavior is observed in both initial velocity and equilibrium binding experiments [3,13].

In the case of classical non-competitive inhibition (double-reciprocal plots intersecting on the abscissa), the percent inhibition produced by a given concentration of inhibitor is independent of substrate concentration. The provides a qualitative assessment of the noncompetitive nature of the inhibition of ATP-ADP exchange by lysine, leucine or phenylalanine. (It should be noted that double-reciprocal plots are not appropriate for the studies presented here because both substrates are varied simultaneously and any linear plot obtained could only result from a

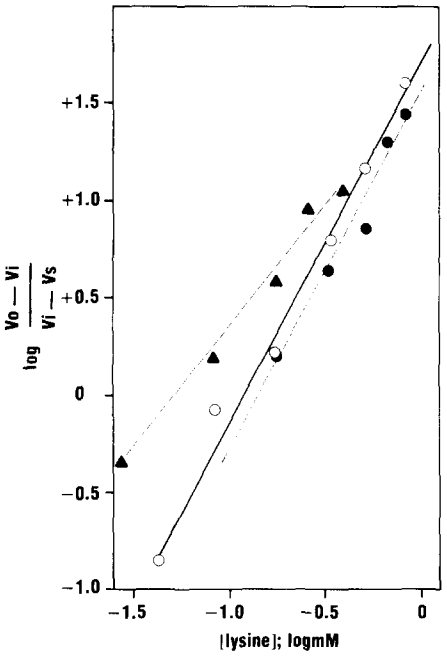

Fig. 1. Hill plot of lysine inhibition of ATP-ADP exchange. The exchange rates were determined as described in the text. ○, the ATP + ADP concentration was 0.86 mM and was established from an initial 10 : 1 ratio of MgATP/aspartate. The Hill coefficient (n_H) is 1.8 and the $I_{0.5}$ is 0.12 mM in this experiment. ●, the ATP + ADP concentration was 6.9 mM and was established from an initial 1 : 1 ratio of MgATP to aspartate. The Hill coefficient is 1.8 and the $I_{0.5}$ is 0.14 mM. ▲, identical to ● except that 1 mM L-leucine was present. The Hill coefficient is 1.2 and the $I_{0.5}$ is 0.052 mM. $I_{0.5}$, the concentration giving half-maximal inhibition, is related to the constant, K, in the Hill equation by the relationship: $K = (I_{0.5})^h$. v_o, v_i and v_s are, respectively, velocities at zero, intermediate and saturating concentrations of lysine. Inhibition by lysine at saturation is >99%.

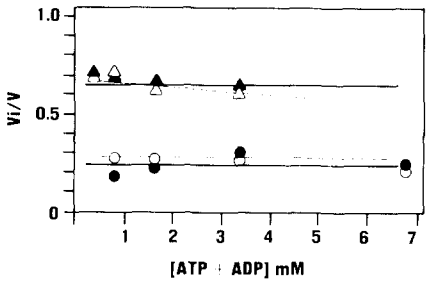

Fig. 2. Influence of substrate concentration and ATP/ADP ratio on inhibition of ATP-ADP exchange by lysine or leucine. Circles, ATP/ADP ratio 0.9 : 1.0. Triangles, ATP/ADP ratio 9 : 1. ●, ▲, lysine, 0.086 mM; ○, △ leucine, 2.6 mM. Rates were measured as described. The ratio of inhibited to uninhibited velocity is plotted vs. total nucleotide concentration.

fortuitous combination of constant terms.) In figs. 2 and 3, it is demonstrated that inhibition by these inhibitors is relatively independent of substrate concentration as predicted for classical noncompetitive inhibition.

In order to establish a higher ADP/ATP ratio we also established equilibrium in reactions initially containing an aspartate concentration 1/50 that of the total nucleotide concentration and ADP in equimolar concentration to ATP. In this way, the extent of reaction of aspartate does not materially perturb the ATP or ADP concentrations and ATP-ADP exchange can be studied at a higher ADP/ATP ratio. The experiments shown in Fig. 2 and 3 include data obtained from this approach. In Fig. 2, inhibition by 0.087 mM lysine or 2.6 mM leucine increases markedly when the ATP/ADP ratio decreases from about 9 : 1 to near 1 : 1. Similarly in Fig. 3, 4.3 mM phenylalanine gives about the same inhibition at the latter ratio as 8.6 mM does at the former ratio. However, inhibition by the saturating concentration of 17.2 mM is the same regardless of ATP/ADP ratio. This increase in sensitivity to inhibition is due to the increased ADP concentration and not the decreased aspartate concentration. This conclusion is based on comparison of data presented in Fig. 1. The data presented in Fig. 1 as open circles were obtained at low aspartate concentrations (1/10 the total nucleo-

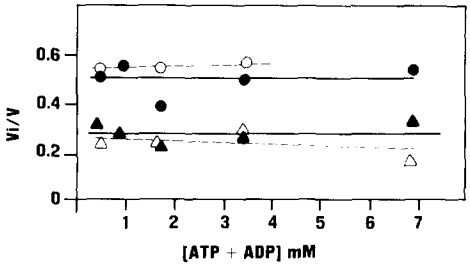

Fig. 3. Influence of substrate concentration and ATP/ADP ratio on inhibition of ATP-ADP exchange of phenylalanine. Concentrations are (○) 8.6 mM, (△) 17.2 mM at an ATP/ADP ratio of 9 : 1 and (●) 4.3 mM, (▲) 17.2 mM at an ATP/ADP ratio of 0.9 : 1.0.

tide concentration) and yet are comparable to the data presented as closed circles which were obtained at an aspartate: total nucleotide ratio of 1 : 1. Thus, the ATP/ADP ratio influences the extent of inhibition but in either case the extent is relatively constant with respect to total substrate concentration. This possibly provides a regulatory linkage between regulation by end products and the energy status of the cell and thereby, could help protect the organism from committing ATP to biosynthetic pathways during periods of energy starvation. This biological advantage has been previously recognized in initial velocity studies of several enzymes [14–16] including this aspartokinase [15].

The results presented here indicate that regulatory characteristics determined by initial velocity techniques (high substrate concentration, low enzyme concentration and zero product) also prevail under conditions for equilibrium isotope exchange rates (lower substrate concentration and product present). While neither condition is identical to the intracellular condition, the fact that aspartokinase displays essentially the same regulatory properties in both provides further justification for the idea that regulatory properties determined in vitro have significance in vivo.

Acknowledgement

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