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REGULATION OF GLYCOLYSIS IN LIZARDS: KINETIC STUDIES ON LIVER PYRUVATE KINASE AND PHOSPHOFRUCTOKINASE FROM *LACERTA GALLOTI*

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Abstract—1. Kinetic studies were carried out on pyruvate kinase and phosphofructokinase from the lizard *Lacerta galloti*.

2. Pyruvate kinase is inhibited by ATP and activated by fructose 1,6-biphosphate giving an hyperbolic saturation curve for ATP without the activator which becomes sigmoidal at saturating concentrations of fructose 1,6-biphosphate, giving a moderate cooperativity with a Hill coefficient of $h = 1.72$.

3. Binding of fructose 1,6-biphosphate to pyruvate kinase was studied as protection effect against thermal denaturation, this being the most suitable ligand tested to avoid the loss of activity.

4. Phosphofructokinase is inhibited by ATP at millimolar range and activated by AMP and by fructose 2,6-biphosphate, AMP being the more efficient activator.

INTRODUCTION

Glycolysis regulation in mammalian liver is a well documentated process, though not totally clarified, and it is commonly accepted that it occurs mainly by changes in PFK† and PK activity, induced by their effectors. In fact, these two enzymes are allosteric in liver, PK being inhibited by ATP and activated by F16P, and PFK being inhibited by ATP at the millimolar range and activated by AMP and F26P among others. We have previously studied (Pérez *et al.*, 1982) PK regulation in rat liver, showing both that inhibition of this enzyme by ATP gives a hyperbolic saturation curve without F16P which becomes sigmoidal at 0.1 mM F16P, and that activation by F16P without ATP is also cooperative. PK has been studied in several species (see Munday *et al.*, 1980 for a review) and results have concluded that its activity is always much greater than PFK in liver and muscle, although its activity *in vivo* must be considerably less than when assayed in optimal conditions. On the other hand, the discovery of F26P as a strong activator of PFK (Van Schaftingen *et al.*, 1980; Uyeda *et al.*, 1981a,b) and the confirmation that concentration of this sugar varies according to the physiological state of the cells (see Hers and Van Schaftingen, 1982 for a review) have again centered the problem of glycolysis regulation on the allosteric effects of these two enzymes.

Glycolysis regulation in muscle is different from that in liver for two reasons: first, muscle pyruvate kinase is not an allosteric enzyme, where neither ATP or F16P regulate its activity, and second, ATP changes cannot occur to the same extent as in liver since near constant ATP concentrations are required to allow the function of the regulation system on contractility by calcium through troponin (Weber and Bremel, 1971; Marston *et al.*, 1980). Thus, our knowledge about glycolysis regulation in muscle is much less than in liver.

Glycolysis is a very ancient metabolic pathway in biological evolution but there are important differences in its later stages, especially with respect to the development of the quaternary structure of enzymes and to adaptation to environmental conditions (Rossman, 1981). Thus, glycolysis regulation could be a useful field for the study of metabolic differences in the evolutionary process among different species.

The studies carried out on glycolytic enzymes from poikilothermic vertebrates have pointed out an adaptative shift in LDH and other isoenzyme patterns dependent on temperature (Hochachka, 1965; see Somero, 1975 for a review). LDH from fish has been extensively studied (Sensabaugh and Kaplan, 1972; Sidell and Beland, 1980 among others) as have other glycolytic enzymes from poikilothermic vertebrates. This dependence on isoenzyme patterns and kinetic parameters on temperature changes according to the environmental conditions (see Prosser, 1973 for a review) has been described for several enzymes in fish (Narita and Horiuchi, 1979; Schwantes and Schwantes, 1982; Somero, 1973; Valkirs, 1978), amphibians (De Costa *et al.*, 1979, 1981; Tsugawa, 1980) and reptiles (Beloff-Chain and Rookledge, 1970). The problem in studying evolution of metabolic pathways is that there are adaptative effects due to the influence

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†Abbreviations used: F16P, fructose 1,6-biphosphate; F26P, fructose 2,6-biphosphate; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PK, pyruvate kinase; F6P, fructose-6-phosphate.

of environmental conditions, such as temperature, on kinetic parameters which do not account for evolutionary but rather adaptative differences. In order to obtain more adequate substantial data of glycolytic enzymes we have studied the cooperativity of regulatory effectors on PK and PFK as the two enzymes which depend on glycolysis regulation. As mentioned above, the liver has been used since glycolysis regulation is more understood in this tissue. Finally, this work has been carried out in lizards, as a species less evolved than mammals, in order to compare its regulatory kinetic parameters with those of rodents. Our results show that all studied effects occur in lizards as in rodents, but to a lesser degree, which could reflect a lesser evolved state nearer to the origin of the regulatory features of ATP and F16P on PK, and ATP, AMP and F26P on PFK.

MATERIALS AND METHODS

Reagents

Porcine heart lactate dehydrogenase type XVIII, rabbit muscle aldolase type I, rabbit muscle glycerophosphate dehydrogenase type I, rabbit muscle triosephosphate isomerase type III, AMP, ADP ATP, NADH, F16P and F26P were obtained from Sigma (St. Louis, MO, U.S.A.); all other reagents were analytical reagent grade, purchased from E. Merck (Darmstadt, F.R.G.) or Koch Light (Colnbrook, Bucks, England).

Biological samples

Male and female lizards (20–30 g), *Lacerta galloti*, endemic species of Tenerife island, were used for experiments 2–5 days after capture, maintained during this time in captivity with feed and water *ad libitum*. Lizard livers were obtained under ether anesthesia from 3–6 animals in each experiment, washed with 0.14 M NaCl, pooled, cooled, chopped and homogenized at 1 g/3 ml in 0.25 M sucrose for PK experiments and in 50 mM Tris–HCl buffer, pH 7.4 with 10 mM ammonium sulfate, 10 mM dithiothreitol, 2 mM EDTA and 1 mM $MgCl_2$ for PFK experiments, by using a Potter–Elvehjeim homogenizer with Teflon pestle, in an ice-cold bath. The homogenates were clarified twice by centrifugation at 27,000*g* in a Sorvall RC-5B centrifuge at 3–4°C for 20 and 10 min, respectively. The resulting supernatants were used immediately for kinetic experiments and their protein concns were determinated by the method of Lowry *et al.* (1951).

Kinetic experiments

All kinetic experiments were developed by a continuous recording method at 340 nm using a Hitachi 100-60 spectrophotometer with a Churchill thermocirculator and a Linear recorder. All kinetic experiments were carried out at 25°C except to study influence of temp on enzyme activity, where experiments were carried out in the 5–50°C temp range. PK reaction was measured as previously described (Perez *et al.*, 1982) by coupling with LDH reaction. The incubation mixtures contained: 0.14 mM NADH, 0.1 M KCl, 2 mM $MgCl_2$, ADP and PEP according to the experiment, 2 U/ml LDH and 50 mM Tris–HCl buffer, pH 7.4. PK activity was assayed at saturating concns of substrates in V_{max} conditions, according to the results of our kinetic experiments, i.e., 5 mM ADP and 20 mM PEP. PFK reaction was measured according to Ling *et al.* (1966) with some modifications, by coupling with aldolase, triose phosphate isomerase and glycerophosphate dehydrogenase reactions. The incubation mixtures contained: 0.14 mM NADH, 25 mM KCl, 5 mM $MgCl_2$, F6P and ATP according to the experiment, 0.1 mM dithiothreitol, 5 mM $(NH_4)_2SO_4$, 0.4 U/ml aldolase, 0.4 U/ml glycerophosphate dehydrogenase, 25 U/ml triosephosphate isomerase and 0.1 M Tris–HCl buffer, pH 7.4. PFK activity was assayed at saturating concentration of F6P (3 mM) and non-inhibitory ATP concn (0.5 mM), in V_{max} conditions, according to the results of our kinetic experiments.

Mixtures of 2 ml containing the appropriate reagents and tissue extracts were incubated in the sample cuvette of the spectrophotometer using as reference the same mixture with all reagents except NADH. Reactions were started by addition to the mixture of the diluted enzymatic extract (100 μl) to obtain 0.8–1.2 protein mg in the incubation mixtures, and NADH decay was recorded during 8–10 min. A good linearity was obtained in the time from 0.8 to 0.1 absorption units, enough to obtain initial velocity values. In experiments to study regulation effects of ATP, AMP, F16P and F26P, about 3 min after triggering the reaction, the regulating reagent, dissolved in the same buffer, was added to the incubation mixture (50 μl) to obtain the appropriate concn in the total of the mixture, recording the new slope of the reaction. In Fig. 1 is shown a schematic diagram of these kinetic experiments.

ATP inhibition on PK was calculated from graphic records by reference to primary rate (before ATP addition) according to the expression

$$\% \text{ inhibition by ATP} = \frac{v - v_{ATP}}{v} \times 100$$

where v is the velocity at saturating ADP concentration (5 mM) and 1 mM PEP before ATP addition, which was a constant value in this series of the experiments, and v_{ATP} is the velocity obtained after ATP addition. ATP inhibition on PK activated by F16P was calculated from graphic records by reference to primary rate (before ATP addition and with 0.2 mM F16P) using

$$\% \text{ inhibition by ATP} = \frac{v_F - v_{F,ATP}}{v_F} \times 100$$

where v_F is the velocity in the same conditions as described above plus 0.2 mM F16P, also a constant value in this series of experiments, and $v_{F,ATP}$ is the velocity obtained after ATP addition. In these experiments ATP saturation for enzyme at 0.2 mM F16P is

$$\bar{y}_{F,ATP} = \frac{v_F - v_{F,ATP}}{v_F}$$

and their fractional saturation values are

$$\frac{\bar{y}_{F,ATP}}{1 - \bar{y}_{F,ATP}} = \frac{v_F - v_{F,ATP}}{v_{F,ATP}}.$$

F16P activation on PK was calculated by reference of values obtained after F16P addition (v_F) as compared with velocity values before F16P addition (v) using

$$\% \text{ activation by F16P} = \frac{v_F - v}{v} \times 100.$$

A similar expression was used to calculate AMP activation on PFK velocity. The results presented in this paper are the means of five experiments, giving a coefficient of variation $(\rho/\bar{x}) \times 100 = 3.2$.

RESULTS

Routine experiments to determine kinetic parameters elicited the data shown in Table 1. The kinetics of PK for both PEP and ADP are demonstrated by hyperbolic saturation curves without ATP, allowing us to obtain K_M values for these substrates from Lineweaver–Burk plots (not shown). The kinetics of PFK for F6P with 0.5 mM ATP are demonstrated by a sigmoidal saturation curve (Fig. 2) of which the cooperativity can be calculated by the Hill plot,

Table 1. Kinetic parameters of lizard liver pyruvate kinase and phosphofructokinase*

	sp. act.†	K_M‡ a§	b§	K_i(ATP)‖	K_a‡‡
PK	0.210	1.12 (5.0)	0.25 (20)	0.51¶/1.2**	7.35 μM§§ 2.1 μM‖‖(F16P)
PFK	0.011	0.75 (0.5)	0.07 (3)	3.0††	0.25 mM (AMP)¶¶

*These results are the means of five experiments, giving a coefficient of variation $(\sigma/\bar{x}) \times 100 = 3.2$.
†μmoles of released product per min, per protein mg. Enzymes assayed in optimal conditions.
‡Apparent K_M values at saturating concns of the other substrate for PK and ATP at non-inhibitory concentration for PFK, obtained from Lineweaver–Burk plots; $S_{0.5}$ value for PFK saturation by F6P. All data are millimolar concns.
§The substrate *a* is PEP for PK and F6P for PFK; the substrate *b* is ADP for PK and ATP for PFK. The saturating concn of the other substrate used in experiments to obtain these values figure in parenthesis.
‖Apparent K_i values as the millimolar ATP concns which account for 50% inhibition.
¶Substrate concns used: 1 mM PEP and 5 mM ADP.
**The same concns of substrates as ¶ plus 0.2 mM F16P.
††Value obtained from the inhibition phase of the saturation curve with 3 mM F6P. See Fig. 3.
‡‡Dissociation constant of enzyme-activator binding from Lineweaver–Burk plot.
§§Substrate concns used: 1 mM PEP and 5 mM ADP.
‖‖The same substrate concns as §§ plus 0.9 mM ATP.
¶¶Substrate concns used 5 mM and 3 mM F6P.

giving a Hill coefficient $h = 2.5$; $S_{0.5}$ for ATP F6P being 0.75 mM. The kinetics of PFK are shown in Fig. 3 in which there are two phases; the first typically hyperbolic, from which the Lineweaver–Burk plot K_M was calculated at saturating F6P concentration (3 mM), and the second which corresponds to ATP inhibition kinetics from which apparent K_i was calculated.

ATP inhibition on PK, shown in Fig. 4, gives a hyperbolic curve at saturating ADP concn (5 mM) and K_M concn of PEP (1.12 mM), when F16P was not present. However, with saturating concns of this sugar (0.2 mM), the kinetic saturation curve becomes sigmoidal, although its cooperativity obtained from the Hill plot is not great, giving $h = 1.72$. In these conditions apparent K_i for ATP, as ATP concn which accounts for 50% inhibition, becomes 1.7 mM from its previous value of 0.5 mM. Thus, F16P activates PK revealing the cooperativity for ATP. Moreover, F16P activates the enzyme both with ATP or without it. This activation gives hyperbolic saturation curves presented in Fig. 5 showing that 0.1–0.2 mM F16P totally saturates the enzyme, but activation constants obtained from Lineweaver–Burk plots (not shown) are different in these two conditions, these being 7.35 μM F6P without ATP and 2.10 μM, with ATP at concn about the value of K_i. Thus, ATP enhances affinity of the enzyme for its activator F16P. There-

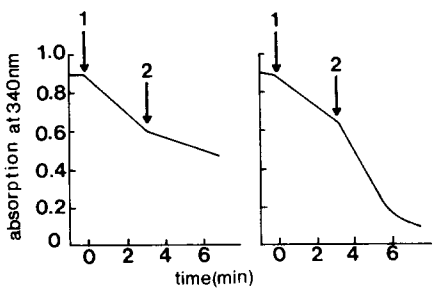

Fig. 1. Graph records of the kinetic experiments. Typical kinetic experiments to study regulation effects on PK and PFK. Soluble fractions of lizard liver were incubated in the cuvette of the spectrophotometer at 25°C (except in experiments to study temp effects, where several temps were used) with appropriate substrates according to the experiment as described under Materials and Methods; absorption decay at 340 nm was continuously recorded obtaining velocity values from slopes in the graph records. Reactions were started by addition of tissue extract (100 μl) to obtain 0.1–0.15 protein mg per ml in the incubation mixture; 3 min after triggering reaction enzyme regulators (ATP or F16P in PK experiments, and AMP or F26P in PFK experiments) were added to the mixture in 50 μl to obtain appropriate concns according to each experiment. In each experiment two slopes were obtained: the first, before modulator addition and the second after it, the two having good linearity which allows us to calculate initial velocity values in all cases. Left: Inhibition effect of ATP on PK; arrow 1, tissue extract addition; arrow 2, ATP addition. Right: Activation effect, i.e., F16P on PK and AMP or F26P or PFK; arrow 1, tissue extract addition; arrow 2, activator addition.

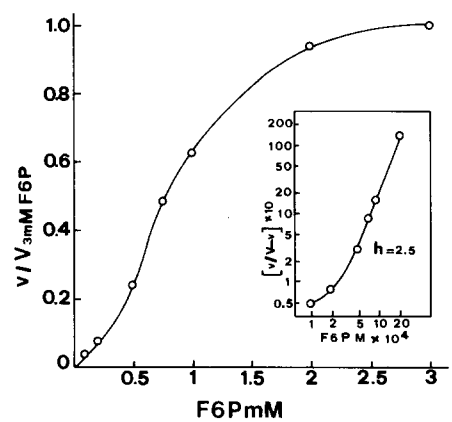

Fig. 2. Kinetics of phosphofructokinase saturation by fructose-6-phosphate. Soluble fractions of lizard liver were incubated for developing PFK reaction as described under Materials and Methods with 0.5 mM ATP in the cuvette of the spectrophotometer at 25°C. Reactions were started by addition of tissue extract (100 μl) to the incubation medium velocity values being obtained from slopes of absorption decay at 340 nm. Sigmoidal form of the curve indicates cooperativity for binding F6P to PFK which is quantified by the Hill plot giving a Hill coefficient $h = 2.5$. According to these data 0.75 mM F6P is the substrate concn which gives half the maximal velocity. The quotients between velocity values of the reaction and velocity value with 3 mM F6P are represented on ordinate axis.

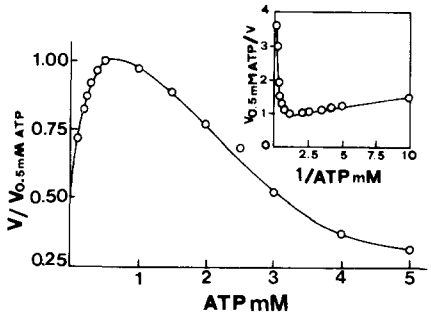

Fig. 3. Kinetics of phosphofructokinase activity vs ATP concn. Soluble fractions of lizard liver were incubated for developing PFK reaction as described under Materials and Methods with 3 mM F6P in the cuvette of the spectrophotometer at 25°C. Reactions were started by addition of tissue extract (100 μl) to the incubation medium and velocity values were obtained from slopes of absorption decay at 340 nm. First phase of this curve gives a linear Lineweaver–Burk plot, the ATP which gives maximal velocity being 0.5 mM and apparent K_M for ATP obtained from these data being 0.07 mM; apparent K_i value for ATP, as ATP concn which accounts for 50% inhibition, can also be calculated, this being 3 mM, in the inhibition phase of the curve. The quotient values between velocity in each case and velocity at 0.5 mM ATP are represented on ordinate axis.

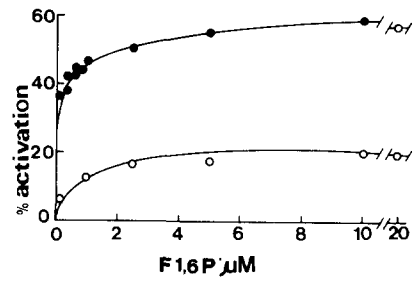

Fig. 5. Activation of pyruvate kinase by fructose 1,6-bisphosphate. Soluble fractions of lizard liver were incubated for developing PK reaction as described under Materials and Methods with 1 mM PEP and 5 mM ADP, without ATP (○) and with 0.9 mM ATP (●). Reactions were carried out in the cuvette of the spectrophotometer at 25°C, started by the addition of tissue extract (100 μl) to the incubation medium; 3 min after triggering the reaction F16P was added (50 μl) to obtain the indicated concns in the incubation mixture. Percent of activation values were obtained from slopes of absorption decay at 340 nm before (v_1) and after (v_2) F16P addition by $(v_2 - v_1) \times 100/v_1$, v_1 being the rate without activator which, in experiments with 0.9 mM ATP (●), corresponds to 75% inhibition with respect to the same value without ATP (○). The activation effect is greater on inhibited enzyme although it is an activation effect of a lower velocity.

fore, F16P is a greater activator with ATP although the enzyme does not recover its velocity, only reaching 80% of its previous rate, a value which corresponds to 60% activation over 50% inhibited enzyme with saturating F16P concentrations. Non-inhibited enzyme, however, shows only 20% maximal activation at the same F16P concentration. This activation effect on PK by binding of F16P is very clear in data from thermal denaturation experiments shown in Fig. 6, where the protection effect of F16P on thermal loss of activity of the enzyme can be seen. In these experiments all other ligands of the enzyme are present in the same conditions but only F16P binding to the enzyme avoids thermal denaturation. In the first range, from 5 to 35°C we have the expected thermal activation and in the second range, at higher temps, there is a loss of activity only when F16P is not included in the incubation mixture, reaching total loss of activity at 50°C; however, with F16P at saturating concentration, all other ligands being at the same conditions, thermal activation is maintained at this temp, which suggests a useful way of purifying the enzyme. Q_{10} value calculated from these data was 2.7.

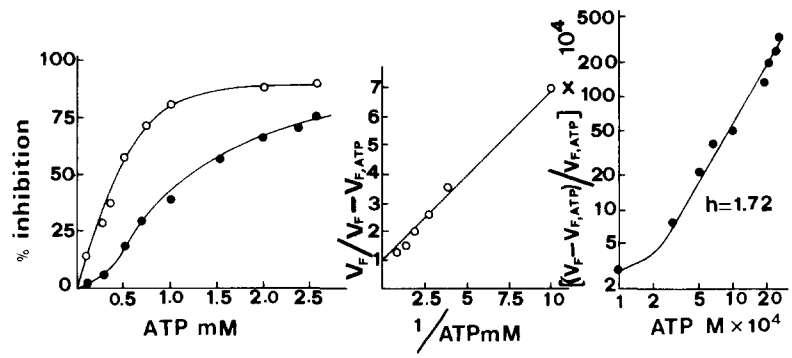

Fig. 4. Inhibition of pyruvate kinase by ATP. Soluble fractions of lizard liver were incubated for developing PK reaction as described under Materials and Methods with 1 mM PEP and 5 mM ADP without F16P (○) and with 0.2 mM F16P (●) in the cuvette of the spectrophotometer at 25°C. Reactions were started by addition of tissue extract (100 μl) to the incubation medium, and 3 min after triggering the reaction ATP was added (50 μl) to obtain the indicated concns in the incubation mixture. Percent of inhibition values were obtained from slopes of absorption decay at 340 nm before and after ATP addition. Apparent K_i values, as ATP concn which account for 50% inhibition in these conditions, are, according to these results, 0.5 mM without F16P and 1.2 with 0.2 mM F16P. Hyperbolic saturation curve for ATP without F16P gives good linearity in the Lineweaver–Burk plot which shows no cooperativity for binding ATP to the enzyme. However, with 0.2 mM F16P a sigmoidal saturation curve is obtained showing a certain cooperativity in the Hill plot, giving $h = 1.72$.

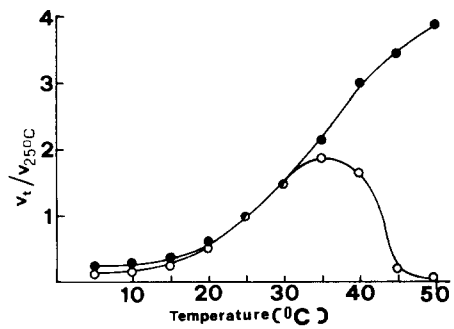

Fig. 6. Protection of fructose 1,6-biphosphate on thermal denaturation of pyruvate kinase. Soluble fractions of lizard liver were incubated for developing reaction as described under Materials and Methods with 1 mM PEP, 5 mM ADP, and 0.5 mM ATP (○) and all previous reagents at same concns plus 0.2 mM F16P (●). Reactions were carried out in the cuvette of the spectrophotometer in the temp range from 5 to 50°C, in different experiments. Velocity values were obtained from slopes of absorbance decay at 340 nm. $v_t/v_{25°C}$ represented in the ordinate axis is the quotient among velocity values obtained in each experiment and the constant value obtained at 25°C.

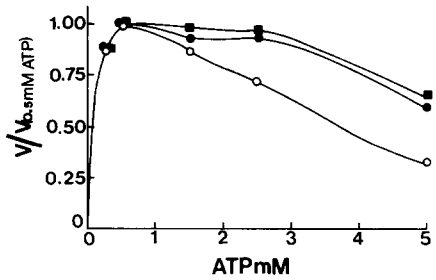

Fig. 8. Fructose 2,6-biphosphate activation of phosphofructokinase. Soluble fractions of lizard liver were incubated to develop PFK reaction as described under Materials and Methods with 3 mM F6P. Reactions were carried out in the cuvette of the spectrophotometer at 25°C started by the addition of diluted tissue extract (100 μl) to the incubation medium and, 3 min after triggering the reaction, F26P was added in 50 μl to the mixture. Velocity values were obtained from slopes of absorbance decay at 340 nm before and after F26P addition. Concentrations of F26P in the incubation mixture are: none (○); 7.5 μM (●) and 15 μM (■). The quotients between velocity values of the reaction and the velocity value with 0.5 mM ATP are represented on ordinate axis.

AMP and F26P activation on PFK are shown in Figs 7 and 8, respectively. It can be seen that AMP is a more efficient activator than F26P, since at the high ATP concn used (5 mM) the enzyme recovers its activity with AMP at saturating concns, but this does not occur with F26P in the same conditions. Kinetics of AMP saturation of PFK is hyperbolic allowing us to obtain its dissociation constant from the Lineweaver–Burk plot giving $K_a = 0.25$ mM. It is important to observe, on the other hand, that, according to our results, F26P does not activate PFK in the ATP concn range of the first phase of the saturation curve, where ATP does not inhibit.

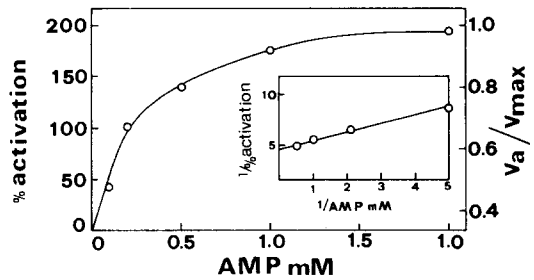

Fig. 7. AMP activation of phosphofructokinase. Soluble fractions of lizard liver were incubated for developing PFK reaction as described under Materials and Methods with 5 mM ATP and 3 mM F6P. In these conditions, ATP produces the maximal inhibition, PFK activity being about 33% of its value at optimal ATP concn (see Fig. 3). Reactions were carried out in the cuvette of the spectrophotometer at 25°C, started by the addition of tissue extract (100 μl) to the incubation medium and, at 3 min after triggering the reaction, AMP was added to the mixture in 50 μl at appropriate concns according to the experiment. Percent of activation values were obtained from slopes of absorbance decay at 340 nm before and after AMP addition. Origin in ordinate axis is the primary rate (before adding AMP) which corresponds to 33% rate with respect to V_{max} (with 0.5 mM ATP, see Fig. 3). V_a is the second rate, after adding AMP. The hyperbolic curve is asymptotic to V_{max}, indicating that AMP can totally eliminate ATP inhibition.

DISCUSSION

Ratio of activities of PK/PFK in the studied lizard is great as found in other vertebrate species including mammals (see Munday *et al.*, 1980 for a review). This high ratio, apparently generalized could be explained in the light of the regulation features of glycolysis based on PK and PFK allosteric regulation, since PK catalyzes one of the last reactions, while PFK acts on the first steps of glycolysis. Thus PFK could be more critical in its changes of activity. The kinetic features we have found in lizard which differ from rat liver are the Hill coefficient of PK for ATP at saturating F16P concn which is 2.84 in rat liver (Pérez *et al.*, 1982) and 1.72 in lizard liver, and the small activation effect of F26P on lizard liver PFK, as compared with rat liver (Hers and Van Schaftingen, 1982). These results suggest that activation of PK by F16P could be an ancient effect in biological evolution but in lesser evolved species than mammals its cooperative binding could be less important, this being increased in the evolution to rodents. With respect to the other point mentioned, it is possible that F26P does not have an important physiological significance in lizards since its effect on PFK activity at saturating concn is very small if compared with activation by AMP. Because these two differences discussed between lizard and rat liver refer to activation effects it could be thought that activation has been achieved or enhanced in the later stages of biological evolution. Further studies on glycolysis regulation patterns could give more information about the evolution of kinetic parameters, this possibly being glycolysis regulation, a promising field for the study of biological evolution.

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