THE INHIBITION OF PYRUVATE KINASE BY ATP

T. Wood

Department of Biochemistry, McGill University, Montreal, Quebec

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A number of authors have reported inhibition of muscle pyruvate kinase by ATP (Meyerhof and Oesper, 1949; McQuate and Utter, 1959; Reynard et al., 1961; Mildvan and Cohn, 1966; Tanaka et al., 1967) and the occurrence of this inhibition has been incorporated into schemes to explain the control of glycolysis in brain (Lowry and Passoneau, 1964; Rolleston and Newsholme, 1967). Some experiments carried out in this laboratory appeared to confirm these findings but further experiments designed to reduce the effects of complexing of magnesium and of pH changes, completely eliminated the inhibitory action of ATP on the forward reaction (PEP + ADP → ATP + pyruvate). A careful scrutiny of the experimental conditions used by various workers who have reported ATP inhibition revealed that this inhibition, attributed by Reynard et al. (1961) to competion with ADP and PEP for the active site of pyruvate kinase, can be accounted for by its magnesium chelating action. In fact, McQuate and Utter (1959) explained the inhibition they found on this basis.

In the present paper, experiments are reported which demonstrate that the inhibition by ATP depends on the level of magnesium in the medium, and the inhibition observed by Reynard et al. (1961) was partially reproduced. However, when the magnesium concentration was raised to a point where all the magnesium-complexing species were saturated with the metal, the inhibition disappeared.

Materials and Methods

Pyruvate kinase activity was measured by following the rate of NADH oxidation in the coupled reaction with lactate dehydrogenase in a Calbiometer (Calbiochem, Los Angeles, 63). Initially, the conditions approximated those described by Czok and

Eckert (1965) but were progressively modified as shown in Table 1. Crystalline rabbit muscle pyruvate kinase (125 I.U./mg, 2 mg/ml) and crystalline lactate dehydrogenase (360 I.U./mg, 5 mg/ml) were obtained from Boehringer Mannheim Corp., New York, and were diluted before use in 1 mg/ml bovine serum albumin. Pyruvate kinase at a dilution of 1 : 400 and lactate dehydrogenase at 1 : 10 were found to be stable for at least 6 hrs at 0°.

In the cuvette was placed 1.50 ml of a buffer solution containing the required amounts of KCl and $MgCl_2$ (in a few experiments $MgCl_2$ was added separately), to this was added the ADP, PEP, ATP (if present), NADH, and water to a volume of 2.00 ml. Diluted lactate dehydrogenase (0.01 ml of 1 : 10) was added and when the optical density had reached a steady value the reaction was started by the addition of pyruvate kinase. Readings of optical density at 340 mμ were taken at 30 second intervals for 10–14 minutes. The optical densities were plotted against time and the initial velocity was calculated from the slope of the linear portion of the plot which usually extended beyond 6 minutes. In some experiments, the pH of the cuvette contents at the end of the run was checked with a pH-meter.

Results and Discussion

The initial velocities of pyruvate kinase under various reaction conditions are listed in Table 1 as optical density units/minute/ml undiluted enzyme (divide by 3.11 to convert to μmoles/min./ml). The results in group (a) demonstrate the expected inhibition by ATP, when a large part of the magnesium is already complexed by EDTA. Group (b) demonstrates the absolute requirement for magnesium, even when the amount of enzyme was increased 400-fold in the absence of extra magnesium no activity could be detected. Group (c) shows the very much greater inhibition by ATP when the magnesium concentration was low and EDTA was absent. In group (d), when the magnesium concentration was raised, a lack of ATP inhibition was observed but the inhibition reappeared when EDTA was added.

An attempt was made to reproduce the inhibition observed by Reynard et al.(1961) but the inhibition obtained (group (e)) was found subsequently to be due to the low acidity of the disodium salt of ATP which reduced the pH of the reaction mixture considerably at

the low buffer concentration used. When the pH of the ATP was adjusted to 7.0, the

Table I

Inhibition of Pyruvate Kinase by ATP

	Rate ODu/min./ml
(a) 46 mM triethanolamine buffer, 8.9 mM EDTA, 67 mM KCl, 7.3 mM $MgCl_2$, pH 7.6 (1)	740
" + 5 mM ATP	90
(b) 50 mM imidazole buffer, 8.9 mM EDTA, 67 mM KCl, 1.1 mM $MgCl_2$, pH 7.5	0
" + 10 mM $MgCl_2$	635
as above using 0.01 ml undiluted enzyme	0
(c) 50 mM imidazole buffer, 12 mM KCl, 1.1 mM $MgCl_2$, pH 7.5	184
" + 5 mM ATP	6
(d) 50 mM imidazole buffer, 67 mM KCl, 7.5 mM $MgCl_2$, pH 7.5	480
" + 5 mM ATP	490
" + 5 mM ATP + 10.3 mM EDTA	20
(e) 10 mM glycylglycine buffer, 100 mM KCl, 10 mM $MgCl_2$, pH 8.5	328
" + 5 mM ATP (2)	58
(f) 20 mM glycylglycine buffer, 100 mM KCl, 10 mM $MgCl_2$, pH 8.5	367
" + 5 mM ATP (3)	340
as above, pH 7.5 + 5 mM ATP (3)	405

All experiments were carried out at 30°, 0.25 mM ADP and 0.37 mM PEP with the addition of 0.01 ml of pyruvate kinase diluted 1 → 400 with 1 mg/ml bovine serum albumin. (1) Conditions of Czok and Eckert (1965); (2) pH of solution was 3.6; (3) pH of solution was 7.0.

inhibition was slight (group (f)). In a further series of experiments (Table 2) the inhibition observed by Reynard et al. (1961) was qualitatively, but not quantitatively, reproduced both at pH 7.5 and 8.5. At a 2 mM concentration of ADP, the amount of magnesium left uncomplexed by the added ATP is insufficient to convert all the ADP present to Mg-ADP, which Melchior (1960) has produced evidence to show is the true substrate for pyruvate kinase - consequently addition of ATP produces a fall in rate. The complexing action of ATP can be overcome by increasing the magnesium concentration and, as shown in Figure 1 the inhibition was reversed. The inhibition was greatest when the magnesium concentration was low and gradually disappeared when the Mg : ATP ratio rose above 1, until at 15 mM magnesiun no inhibition could be detected at all.

Published values for the formation constants of $Mg\ ADP^-$ and $Mg\ ATP^{2-}$ are of the order of 2×10^3 and 2×10^4 respectively (Melchior, 1965; Mildvan and Cohn, 1966). Melchior (1965) has pointed out that in the presence of 200 mM potassium the greater

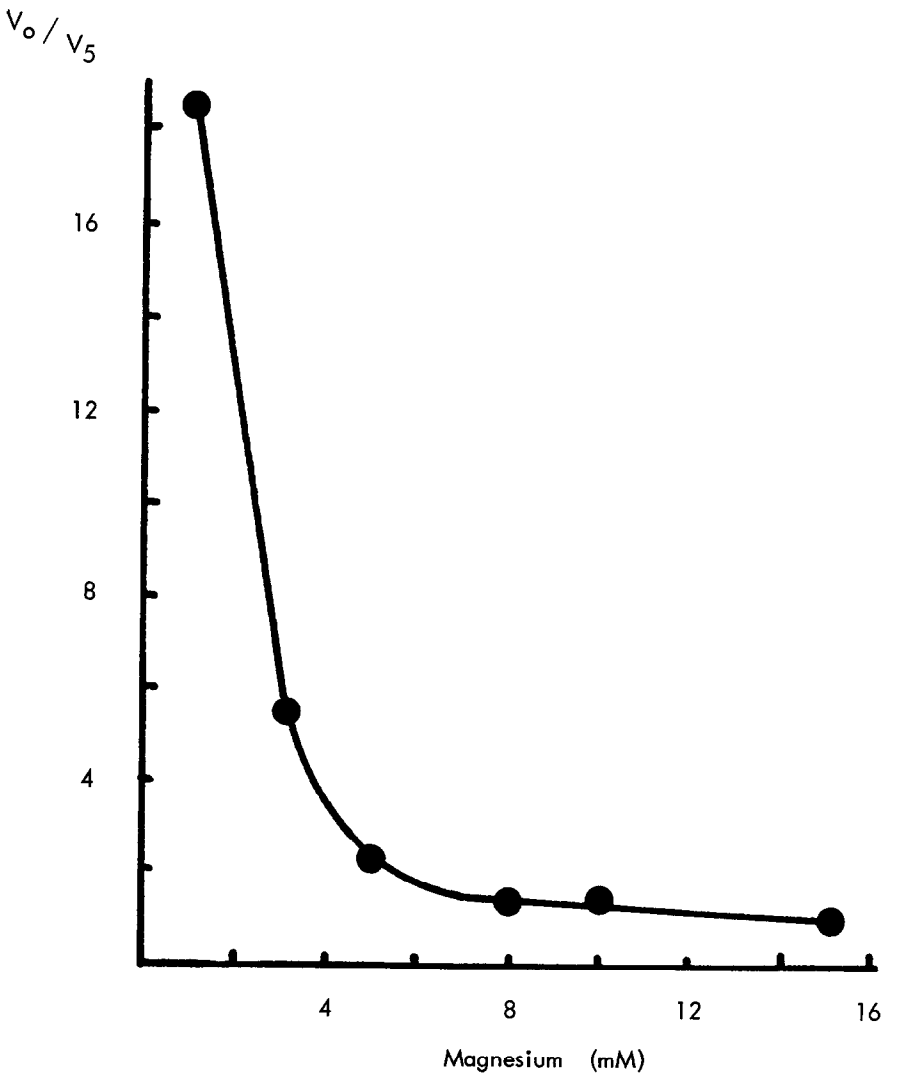

Figure 1. Inhibition of pyruvate kinase by 5 mM ATP at various concentrations of added magnesium.

Conditions: 50 mM imidazole buffer, 100 mM KCl, 0.37 mM PEP, 0.25 mM ADP, pH 7.5, 30°. V_o = velocity without ATP, V_5 = velocity in presence of 5 mM ATP.

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part of the ATP in solution is in the form of its magnesium salt when an equimolecular amount of magnesium is present, while for ADP a 10 to 20-fold excess of magnesium is necessary. On this basis, a 2 mM concentration of ADP would require at least 20 mM magnesium to convert most of it into Mg-ADP, the true substrate for pyruvate kinase. If ATP

Table 2

Inhibition of Pyruvate Kinase by ATP Under the Conditions of Reynard et al. (1961) at 30°.

a) 10 mM glycylglycine buffer, 100 mM KCl, 10 mM $MgCl_2$, 0.37 mM PEP, 2 mM ADP

	Rate (ODu/min./ml)	V_o/V_5*	V_o/V_5†
pH 8.5 no ATP	893	2.6	1.3
pH 8.5 + 5 mM ATP	346		
pH 7.5 no ATP	758	2.0	-
pH 7.5 + 5 mM ATP	382		

b) 10 mM glycylglycine buffer, 100 mM KCl, 10 mM $MgCl_2$, 0.12 mM PEP, 0.2 mM ADP

pH 8.5 no ATP	300	1.5	7.0
pH 8.5 + 5 mM ATP	200		

*V_o/V_5 = rate without ATP / rate with 5 mM ATP. † V_o/V_5 as calculated from the results of Reynard et al. (1961).

is added and complexes with an equal amount of magnesium then the concentration of the catalytically active Mg-ADP species will be reduced and the rate can be expected to fall. The conditions used by a number of workers who have reported ATP inhibition are tabulated in Table 3. It seems clear that the results of Tanaka et al. (1967), Mildvan and Cohn (1966), Lowry and Passoneau (1964), and the first series of Reynard et al. (1961) can be explained by a competition of ATP with ADP for the magnesium present. Furthermore, Lowry and Passoneau also had 5 mM phosphate present (formation constant for $MgHPO_4$ = 76) which would further reduce the available magnesium. An explanation in the above terms is more difficult when it comes to explaining the results of Reynard et al. in the second series listed in Table 3 when inhibition was observed when the ADP concentration was as low as 0.2 mM. In my own experiments (Table 2), only a small inhibition could be obtained under similar conditions, however it is worth noting that the buffering capacity of their system was very low and the authors make no mention of precautions to ensure that the pH of the mixture was not changed by the addition of ATP with a resulting fall in rate.

Table 3

Conditions under which ATP inhibition of pyruvate kinase has been measured.

Authors	Buffer and temperature	$[K^+]$ mM	[PEP] mM	[ADP] mM	$[Mg^{++}]$ mM	[ATP] mM	K_i for ATP mM
Mildvan and Cohn (1966)	50 mM tris, pH 7.5, 29°	100	0.01-0.1	2	4	1.25, 2.5	0.33
Reynard et al. (1961)	(i) 10 mM glycylglycine, pH 8.5, 0°	100	0.05-0.4	2	10	0-7.5	0.12
ibid	(ii) 10 mM glycylglycine, pH 8.5, 0°	100	0.1	0.2	10	0-7.5	0.14
Lowry and Passoneau (1964)	20 mM imidazole*, pH 7.0, 28°	150	0.002-0.02	0.14	5	3	1.0
Tanaka et al. (1967)	50 mM tris, pH 7.5, 25°	100	2+	2+	5	1-10	3.5

* K^+ present at potassium acetate, 5 mM K_2HPO_4 also present, enzyme was brain pyruvate kinase.

+ PEP and ADP concentrations not clear from the published account but believed to be 2 mM as in the other experiments described.

It is significant that although the values of the Michaelis constants for ADP and PEP reported by different workers lie in a narrow range (Km for ADP = 1.26×10^{-4} to 2.7×10^{-4} M, Km for PEP = 3.2×10^{-5} to 7.5×10^{-5}), their values for K_i (ATP) cover a 30-fold range (Table 3) suggesting that the inhibition observed was not a true competitive inhibition as suggested by Reynard et al. In fact, Lowry and Passoneau in their experiments with the brain enzyme stated quite specifically that ATP was not competitive with either substrate.

Summary

The inhibition of pyruvate kinase by ATP was investigated under various conditions. It was concluded that the inhibition reported by previous workers was attributable to the magnesium complexing properties of ATP and it was shown that the inhibition was abolished completely when sufficient magnesium was added.

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