COMPETITIVE INHIBITION OF PHOSPHORIBULOKINASE BY AMP

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The inhibition of ATP-dependent CO_2 fixation by AMP has been observed in cell-free extracts of Thiobacillus thioparus, T. novellus, Chromatium D, spinach, and T. ferrooxidans (Johnson and Peck, 1965; Mayeux and Johnson, 1966; Johnson, 1966; and Gale, 1966). The locus of the inhibition is apparently phosphoribulokinase and it has been suggested (Johnson, 1966) that the mechanism involves some direct action by AMP on the enzyme, which is not competitive inhibition. The present studies conducted on cell-free extracts of T. ferrooxidans indicate that the inhibition of CO_2 fixation by AMP is of a competitive nature between AMP and the normal substrate ATP for the phosphoribulokinase enzyme.

MATERIALS AND METHODS

Thiobacillus ferrooxidans was grown in continuous culture as described by Shafia and Beck (1964). The cells were harvested on a Sharples centrifuge, separated from insoluble ferric deposits by differential centrifugation, washed approximately 4 times in 0.01N sulfuric acid, and finally washed 3 times in distilled water. Eight to 10 grams of cells, suspended in distilled water were shaken with approximately 5 grams each of Amberlite resins IR-45 and IR-120 for 10 minutes at ice bath temperatures. The cell suspension was decanted from the resins, re-centrifuged, and the cells suspended

in 20 ml of distilled water. Extracts were prepared by sonic oscillation of the treated cells for 5 minutes with a Branson Model S-75 sonifier. Approximately 5 grams of powdered glass were added to the cell suspension during sonic treatment to aid breakage. Whole cells and debris were removed by centrifugation at 17,000 X G for 30 minutes. The extracts were either used without further treatment, or following dialysis for 18 hours against a 1 liter volume of 0.03M tris-HCl buffer at pH 8.0, containing 10^{-4}M EDTA and 5×10^{-4}M GSH, with the bath being changed once during the dialysis procedure.

The reaction mixture, consisting of tris-HCl buffer, ATP, R-5-P, $MgCl_2$, labeled sodium bicarbonate, and extract, was placed in a 5 ml vaccine vial, flushed with nitrogen gas, stoppered, and placed in a Dubnoff metabolic shaking incubator at 30°C. Samples of 0.3 ml, taken by tuberculin syringes, were placed in stainless steel planchettes, acidified, and evaporated to dryness. Acid-stable products of fixation were assayed by counting on a Tracerlab thin-window flow counter.

EXPERIMENTAL RESULTS

The rate of CO_2 fixation, as well as the final level of fixation, were determined by limiting amounts of either ATP or ribose-5-phosphate. (Gale and Beck, 1966) The addition of AMP to reaction mixtures containing a relative excess of ATP resulted in little, if any, inhibition of fixation. (See Table 1) Less than 10% inhibition at 60 minutes is seen with AMP:ATP ratios approaching 1. On the other hand, in those reaction mixtures where ATP is present in limiting amounts, as seen in Table 2, the addition of AMP resulted in significant inhibition. As the amount of AMP was increased, the degree of inhibition also increased.

Table 1. Effect of AMP on ATP-dependent CO_2 fixation by crude extracts of T. ferrooxidans: excess ATP

ATP μmoles	AMP μmoles	μmoles CO_2 fixed @ 30 min.
0	0	0
5	0	2.72
5	1	2.66
5	2	2.65
5	3	2.50
5	5	2.54

Each reaction vessel contained in a volume of 3.0 ml: 250 μmoles Tris-HCl, pH 8.0; 3 μmoles R-5-P; 20 μmoles $MgCl_2$; 80 μmoles $NaHCO_3$ (sp. act. = 11,000 cpm/μmole); 2.4 mg extract protein.

Table 2. Effect of AMP on ATP-dependent CO_2 fixation by crude extracts of T. ferrooxidans: limiting ATP

ATP μmoles	AMP μmoles	μmoles CO_2 fixed @ 30 min.
1	0	1.32
1	1	.94
1	3	.78
1	5	.73
1	10	.64

Conditions were listed under Table 1.

By holding the ATP at a limiting concentration, as in the kinetic study shown in Fig. 1, the action of phosphoribulokinase becomes the rate-limiting step in the process of CO_2 fixation, and the fixation of CO_2 is merely a measure of the amount of ribulose-1, 5-diphosphate formed by this critical enzyme.

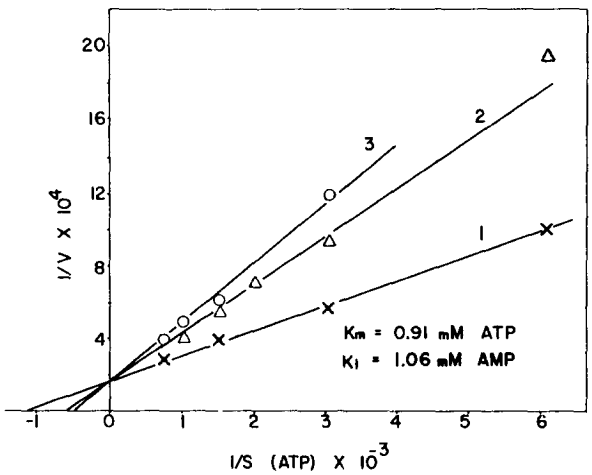

Figure 1. Competitive inhibition of ATP-dependent carbon dioxide fixation by AMP in dialyzed extracts of Thiobacillus ferrooxidans. Reaction mixtures contained in a volume of 3.0 ml: 250 μmoles tris-HCl, pH 8.0; 5 μmoles ribose-5-phosphate; 20 μmoles $MgCl_2$; 80 μmoles $NaHC^{14}O_3$ (sp. act. = 11,000 cpm/μmole); 6 mg extract protein; and ATP as indicated. Line 1 was without inhibitor. Line 2 contained 1.67 mM AMP; line 3 contained 3.3 mM AMP. Velocity is expressed in terms of cpm of a 0.3 ml sample per hour.

The effect of increasing concentration of ATP on rate of CO_2 fixation is seen in line 1. The value of Km is seen to be 0.91×10^{-3}M for ATP, which is in good agreement with data for this enzyme obtained from other autotrophic forms. Maximal velocity under these conditions was calculated to be equivalent to 0.95 μmoles CO_2 fixed per hour per mg of extract protein.

The addition of 1.67 mM and 3.3 mM AMP to the reaction vessels in this kinetic study are seen in lines 2 and 3, respectively. The Lineweaver-Burk plots reveal typical competitive inhibition between AMP and the normal substrate for phosphoribulokinase, ATP. The inhibitor constant, as determined from this figure is 1.02×10^{-3}M, which is of the same order of magnitude as the Km for ATP.

The competitive inhibition by AMP of phosphoribulokinase in T. ferrooxidans may serve in resting cells to resist complete depletion of ATP

pools by continued rapid fixation of carbon dioxide. Inhibiting concentrations of AMP may arise in such resting cells by the action of myokinase on accumulated ADP (Gale, 1964), a process which is probably intimately associated with the maintenance of a certain level of ATP within the cell in the absence of active metabolism.

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