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ADENYLATE KINASE FROM *THIOBACILLUS NEAPOLITANUS*

UNIQUE PROPERTIES, POSSIBLY DESIGNED TO SERVE A UNIQUE METABOLIC FUNCTION

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SUMMARY

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) from *Thiobacillus neapolitanus* was purified to near homogeneity as evidenced by the observance of only 2 protein bands in the final preparation after disc gel electrophoresis at pH 8.3. This purified preparation was investigated to determine if the enzyme's unique role in this organism might be reflected in unusual properties or sensitivities. Mol. wt 23 500; pI, pH 4.2; $K_{m(ADP)}$, $5.5 \cdot 10^{-4}$ M; $K_{m(ATP)}$, $1.1 \cdot 10^{-4}$ M; $K_{m(AMP)}$, $1.3 \cdot 10^{-4}$ M; optimal pH (forward), pH 10.0; optimal pH (reverse), pH 8.0; optimal [Mg^{2+}] (forward) = 1/2 [ADP], and reverse = 1/2 [ATP]; were determined. When compared with the properties of highly purified enzymes from other sources the forward optimal pH, optimal Mg^{2+}:ATP ratio and a protective association of the enzyme with endogenous cytochrome *c* were found to be unusual. The highly alkaline pH optimum for the conversion of ADP → ATP parallels that of 9.5 for cytochrome *c*-coupled APS reductase, another key enzyme of the sulfur oxidation pathway. It is suggested that the inhibition of the reverse reaction by a high Mg^{2+}:ATP ratio may insure the continued operation of the forward reaction and the sulfur oxidation pathway when the free ATP concentration is low and the Mg^{2+} concentration high. The enzyme appears to respond sharply to changes in pH, Mg^{2+} concentration and the presence of endogenous cytochrome *c*. Future investigation of other adenylate kinases which operate far from equilibrium may reveal comparable sensitivities.

INTRODUCTION

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) is generally assumed to operate at equilibrium[1–3]. The enzyme has even been implicated in the

Abbreviations: MES, 2-(*N*-morpholino)-ethane sulfonic acid; TES, *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; CAPS, cyclohexylaminopropane sulfonic acid.

maintenance of a constant intracellular energy charge[4]. Yet in some instances, *i.e.* fetal rat liver[5] and ascites tumor cells[6], careful measurements indicate that the enzyme does not always maintain equilibrium. Further, in some species of *Thiobacilli*, in order for the enzyme to perform a vital physiological function it is imperative that the enzyme not be near equilibrium. In these organisms adenylate kinase converts the final product of the sulfur oxidation pathway, ADP, to the usual energy storage form of the cell, ATP. This pathway supplies at least 40% of the cell's energy requirements[7]. Because of this directional preference, the enzyme from *Thiobacillus neapolitanus* was selected for investigation. It was proposed that the unusual role of adenylate kinase in this organism might be reflected in some unusual enzyme properties, or sensitivities. These properties in turn might elucidate the enzyme's function in this and other instances where it operates as a non-equilibrium enzyme.

MATERIALS AND METHODS

Cultures

T. neapolitanus (obtained from W. Vishniac) was grown as previously described[8]. The cells were harvested during the exponential growth phase with a Sharples continuous flow centrifuge, with a yield of 0.5 g/l. The packed cells were frozen at −60 °C.

Cell extraction and initial purification

8 g of cells were suspended (20%, w/v) in 0.05 M Tris–HCl, pH 7.0, washed twice in the same buffer and ruptured under 20 000 lbs/inch^2 in a French pressure cell. After a few crystals of deoxyribonuclease were added, the extract was centrifuged at 2000 × *g* for 10 min. The resultant supernatant was designated as the crude cell extract and served as the original reference point in later purification studies. The crude extract was further centrifuged at 20 000 × *g* for 30 min. The supernatant (20_s30) was again centrifuged at 144 000 × *g* for 120 min, yielding a supernatant fraction, 144_s120, or soluble cell fraction, and a pellet, 144_p120.

After the addition of β-mercaptoethanol (2 mM), the pH of the soluble cell fraction was adjusted to 4.5 with 2 M acetic acid. After centrifugation at 20 000 × *g* for 20 min $MnCl_2$ (0.05 vol. of a 1.0 M solution) was routinely added to the neutralized acid-soluble fraction and the supernatant fraction containing adenylate kinase activity was saved.

Isoelectric focusing

After dialysis this fraction was subjected to isoelectric focusing in an LKB 8100 Ampholine isoelectric focusing apparatus essentially according to Hoglund[9] initially employing ampholytes in the pH range 3–10, and subsequently 3–6.

Ion exchange chromatography

The $MnCl_2$-treated fraction was dialyzed overnight against 0.01 M potassium phosphate, pH 6.0, containing 1 mM β-mercaptoethanol and was applied to a column of DEAE-Sephadex A-25 (Pharmacia, 1.5 cm × 50 cm) equilibrated with the same buffer. After non-adsorbed proteins were washed through, the enzyme was eluted with a linear salt gradient generated by mixing 300 ml each of 0.15 M and 0.5 M KCl

in the starting buffer which also contained 10^{-4} M AMP. The flow rate was 13 ml/h. The peak fractions were pooled and concentrated by ultrafiltration in cytochrome *c*-coated dialysis bags as described by Callaghan[24].

Molecular sieve chromatography

The concentrated enzyme preparation (5–7 ml) was applied to a column of Sephadex G-75 (Pharmacia, 2 cm × 45 cm) equilibrated with 0.05 M Tris–HCl, pH 7.5, containing 0.1 M KCl. The enzyme was eluted with the same buffer at an upward flow rate of 15 ml/h. The column was prestandardized with proteins of known molecular weight, according to Andrews[10].

The peak fractions were pooled, concentrated by ultrafiltration in cytochrome *c*-coated bags and stabilized by the addition of 0.1% bovine serum albumin. This fraction was used in all kinetic studies.

Disc gel electrophoresis

Electrophoresis of the variously purified fractions was conducted on 7% gels of polyacrylamide at pH 8.3 in Tris–glycine buffer, essentially according to Davis[11] as modified by Shuster[12]. The gels were stained for protein with Coomassie Brilliant Blue (Sigma). Other gels were assayed for adenylate kinase activity according to Rosalki[13].

Protein determinations

Protein was determined according to Lowry *et al.*[14].

Assays of enzymatic activity

Coupled enzymatic activity. In all purification studies, coupled enzymatic assay systems were used to measure adenylate kinase activity. In the forward direction (2 ADP → ATP + AMP) the production of ATP was measured spectrophotometrically essentially according to Oliver[15]. In the reverse direction (ATP + AMP → 2 ADP) the production of ADP was measured spectrophotometrically according to Bucher and Pfleiderer[16].

Direct assay. For all kinetic studies a direct spectrophotometric assay was employed. Adenylate kinase was incubated in 0.5 ml of reaction mix consisting of 0.04 M Tris–HCl, pH 8.5, $MgCl_2$, and substrate for 30 s. The Mg^{2+} concentration was varied to be maintained at 1/2 the ADP or ATP concentration. The reaction was stopped by addition of 0.2 ml of 0.25 M $HClO_4$. The solution was filtered through a Swinnex millipore syringe filter attachment containing HAWP (0.45 μm) filter paper (Millipore) to irreversibly inactivate the enzyme. It was determined that the adenylate nucleotides were quantitatively recovered after filtration. An aliquot was neutralized with 10% NaOH and 1.0 M Tris–HCl, pH 7.6. Alternatively the reaction was terminated by plunging the reaction tube into a vigorously boiling water bath and heating for 5 min. The ATP content of an aliquot from either procedure was then determined essentially according to Williamson and Corkey[17] but modified to allow spectrophotometric determination. In the reverse reaction the ADP content of an aliquot was determined also according to Williamson and Corkey[17].

pH optima

These assays were conducted using saturating concentrations of substrates and

optimal $MgCl_2$ concentrations (as determined at pH 8.5). The final concentration of each buffer used was 0.04 M. The buffers used were: 2-(*N*-morpholino)-ethane sulfonic acid(MES)–NaOH in the pH range 5.5–6.5; *N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid(TES)–NaOH in the pH range 6.5–8.0; Tris–HCl in the pH range 7.5–9.0; and cyclohexylaminopropane sulfonic acid(CAPS)–NaOH in the pH range 9.5–12.

Arrhenius plots

These plots were derived from the data obtained by preequilibrating buffer (0.04 M Tris–HCl, pH 8.5), $MgCl_2$ and substrates at optimal concentrations (as determined at 25 °C) to specified temperatures. 30 s after the addition of adenylate kinase, the reaction was terminated by the addition of $HClO_4$. The Arrhenius energies of activation were obtained from the slopes of the log v *vs* $1/T$ (°K) plots.

K_m determinations

These assays were performed in 0.04 M Tris–HCl, pH 8.5, in both directions. The Mg^{2+} concentration was varied with the ADP or ATP concentration to be maintained at the optimal ratio. The parameters were calculated from the data according to Florini and Vestling[18]. All K_m values given in this text are apparent K_m values.

Purification of substrates

Any given adenylate nucleotide purchased from Sigma contained from 3–5% contamination with other adenylate nucleotides. These nucleotides were, therefore, purified shortly before use on columns (1 cm × 90 cm) of DEAE-Sephadex A-25 which had been converted to the acetate form according to Caldwell[19]. The separated nucleotides were concentrated by lyophilization, and precipitated by the addition of an excess of barium acetate at alkaline pH at 0 °C. The nucleotides were converted to the free-acid form with Dowex-50 cation exchange resin and then to the sodium salt by neutralization with NaOH. The concentration of each nucleotide was determined enzymatically after appropriate dilution, according to Williamson and Corkey[17]. Contaminating adenine nucleotides could not be detected enzymatically in any of the preparations.

RESULTS

Cell fractionation and enzyme purification

Adenylate kinase activity was detected exclusively in the soluble cell fraction, 144_S120. The small amounts of activity found in the particulate fractions, 20_P30 and 144_P120, were released into the soluble phase when these two pellets were resuspended in buffer and recentrifuged. The isoelectric point of the enzyme was determined to be pH 4.2 from three determinations. The recovery of activity was 5–30% of that applied and thus was not practical for purification purposes. Addition of β-mercaptoethanol (0.01 M) to the ampholyte solutions or of various metal ions to the enzyme fractions failed to improve recovery or restore activity. Investigation of the enzyme's pH stability (Fig. 1) revealed relative instability of the enzyme at pH values close to its pI for prolonged periods but relative stability around pH 6.0. Therefore adenylate kinase was subjected to ion-exchange chromatography at pH 6.0.

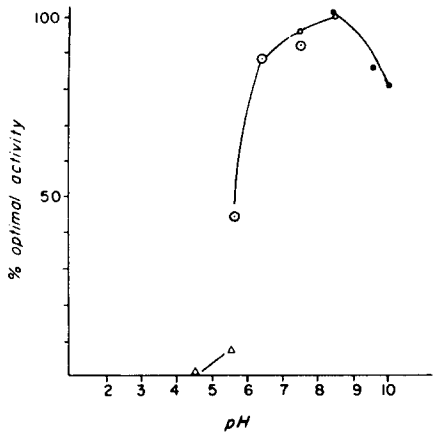

Fig. 1. pH stability of adenylate kinase. Aliquots of the $MnCl_2$-treated fraction were incubated for 24 h in buffers of the designated pH. ●—●, glycine–NaOH; ○—○ Tris–HCl; ○—⊙, sodium phosphate; △—△, sodium acetate.

A major portion of the cell protein was removed either before or after the elution of adenylate kinase. The peak fractions were pooled by ultrafiltration in cytochrome *c*-coated dialysis bags. Unless the tubing was so coated a decrease in the purification index was obtained in the next step in purification, due to a tremendous loss of activity during concentration. The concentrated enzyme preparation was then applied to a column of Sephadex G-75. The elution profile is shown in Fig. 2. The presence of more than one species of protein in the pooled DEAE-Sephadex fractions was revealed by their separation on this column. A protein peak was eluted at the void volume of the column, and another peak with an attendant shoulder was eluted coincident with adenylate kinase activity.

The standard molecular weight curve derived using proteins of known molecular weights is shown in Fig. 3. From the elution volume of adenylate kinase its molecular weight was estimated to be 23 500 (± 10%).

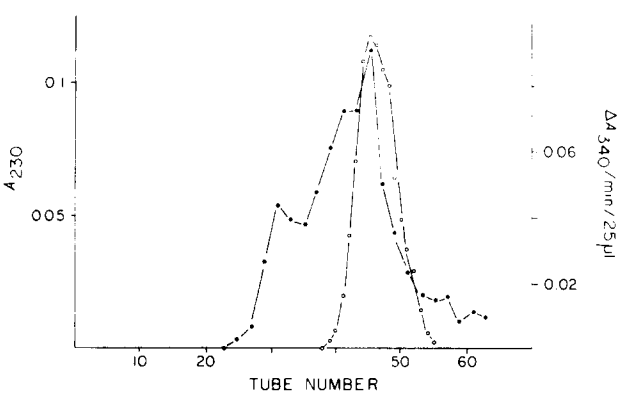

Fig. 2. Sephadex G-75 chromatography of ion-exchange purified adenylate kinase. The concentrated fractions from ion exchange chromatography at pH 6.0 (Fig. 3) were applied. The column was eluted with 0.05 M Tris–HCl, pH 7.5, containing 0.1 M KCl. Fractions of 2.5 ml were collected. ●—●, $A_{230\ nm}$; ○—○, adenylate kinase activity.

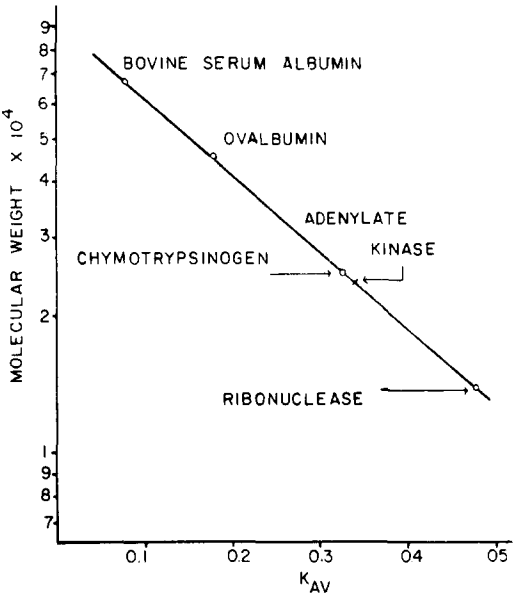

Fig. 3. Standard curve for molecular weight determination on Sephadex G-75. The K_{AV} for proteins of known molecular weight was plotted against the logarithm of the molecular weight of the various proteins to obtain a standard curve. The molecular weight of adenylate kinase as estimated by this curve is 23 500 (± 10%).

The purification scheme of adenylate kinase is summarized in Table I. The progress in purification at each step was monitored by polyacrylamide disc gel electrophoresis. The results of this procedure are shown in Fig. 4. In Fig. 4 Gel B illustrates the banding produced by the $MnCl_2$ fraction (0.34 mg protein), Gel 2 the concentrated DEAE-Sephadex, pH 6.0 fractions (0.23 mg protein), and Gel 3 the concentrated Sephadex G-75 fractions (0.2 mg protein). The great reduction in protein components after ion-exchange chromatography is apparent although the preparation still contains four or five protein bands. After Sephadex G-75 chromato-

TABLE I

PURIFICATION OF ADENYLATE KINASE

The $MnCl_2$ dialysate refers to the supernatant fraction after $MnCl_2$ treatment and after dialysis against phosphate buffer, pH 6.0. Sephadex G-75 refers to the pooled eluant fractions from Sephadex G-75 chromatography; DEAE, pH 6.0, refers to the pooled fractions eluted from DEAE-Sephadex at pH 6.0. A unit of enzyme is that amount of enzyme which produces 1 nmole of product/min. Specific activity refers to the number of units/mg protein. The purification index is the ratio of the obtained specific activity to the specific activity of the crude extract.

Fraction	*Specific activity*	*Purification index*	*Percent recovery*
Crude	139	—	—
144_8120	429	3	90
Acid-soluble	579	5	73
$MnCl_2$ dialysate	2 373	17	61
DEAE, pH 6.0	10 494	74	27
Sephadex G-75	79 550	560	9

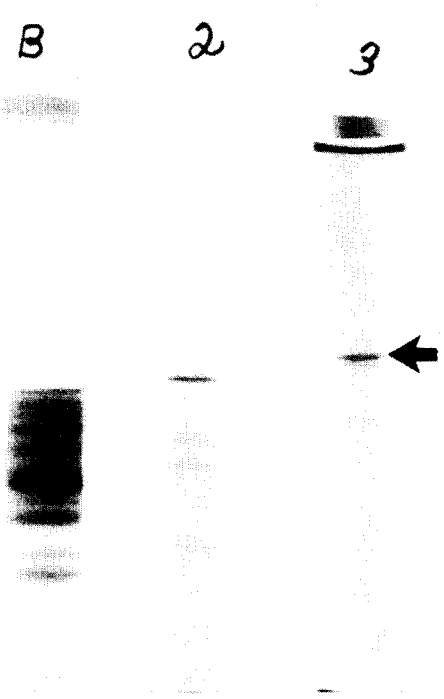

Fig. 4. Disc gel electrophoresis of adenylate kinase during the final stages of purification. B, $MnCl_2$-treated acid-soluble fraction; 2, Ion exchange (pH 6) fraction; 3, Sephadex G-75 fraction. The origin of the gel is at the bottom and the fastest migrating band in each gel is the tracking dye (arrow).

graphy, only two or three protein components could be seen in the enzyme preparation. It was recognized however that it could contain minor components below the limits of detection of this method.

Upon rechromatography of the final purified fraction on Sephadex G-75, the lower band was reduced in intensity relative to the upper band suggesting that the upper, smaller protein is in fact adenylate kinase (such rechromatography resulted in a 75% loss of activity and thus was not routinely used). All attempts to detect adenylate kinase by the activity stain method of Rosalki[13] failed with the purified enzyme. The only samples which would react in the activity assay were those that still contained the soluble cellular cytochrome components (which migrated immediately behind adenylate kinase), suggesting that the purified enzyme had lost activity due to oxidation no longer prevented by the cellular cytochrome during electrophoresis.

The enzyme preparation containing two protein bands was routinely used in the kinetic studies. This preparation was determined to be free of pyruvate kinase, hexokinase, 3-phosphoglycerate kinase and ATPase. Absence of other major competing enzymes was inferred by the virtual superimposability of the Arrhenius plots (Fig. 5) of the forward and reverse reactions. The lines fitted by least squares to the Arrhenius plots were nearly identical in slope. The presence of any major competing enzyme in either reaction would presumably have produced greater variation.

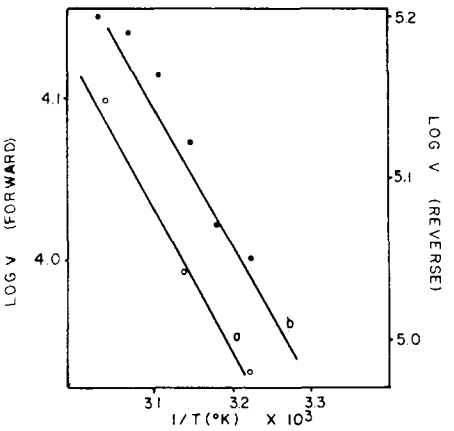

Fig. 5. Arrhenius plots. Forward and reverse reactions. The temperature is given in °K. The slopes of the lines were determined by least squares analysis. Line a is the Arrhenius plot of the data obtained in the forward direction. The apparent E_a calculated from the slope of this line was 4.2 kcal/mole per °K under the conditions specified (pH 8.5, 30 s incubation). Line b is the Arrhenius plot of the data obtained in the reverse direction. The corresponding apparent E_a in this direction (pH 8.5, 30 s) was 4.3 kcal/mole per °K.

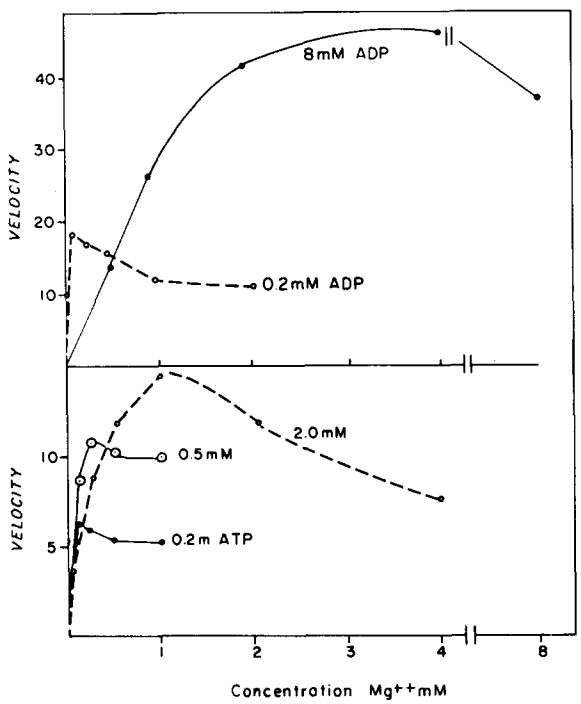

Fig. 6. The effect of Mg^{2+} concentration on the velocity of the forward and reverse reactions. Velocity is given in μmoles ATP produced or utilized/min per mg protein. The Mg^{2+} concentration was varied at 2 or 3 substrate concentrations at pH 8.5.

Enzyme properties

The adenylate kinase reaction velocity when measured at specific time intervals appeared to decrease slightly after 1 min time. An assay period of 30 s was subsequently used to insure measurement of initial velocities. The reaction velocity was found to be directly proportional to enzyme concentration in either direction when 15–150 μg protein were employed.

The effect of Mg^{2+} concentration upon the velocity of the forward reaction was studied at 2 ADP concentrations. All $MgCl_2$ solutions were standardized by the method of Mohr[20]. The upper curve of Fig. 6 represents velocity determinations at near-saturating levels of ADP or 8 mM. The maximum rate in this case occurred when the Mg^{2+} concentration was 4 mM or 1/2 the ADP concentration. In the lower curve when ADP was limiting (0.2 mM or less than 1/2 K_m), the maximum velocity occurred at 0.1 mM $MgCl_2$ concentration, again 1/2 the ADP concentration.

The pH optimum for the forward reaction is depicted in Fig. 7. Optimal adenyl-

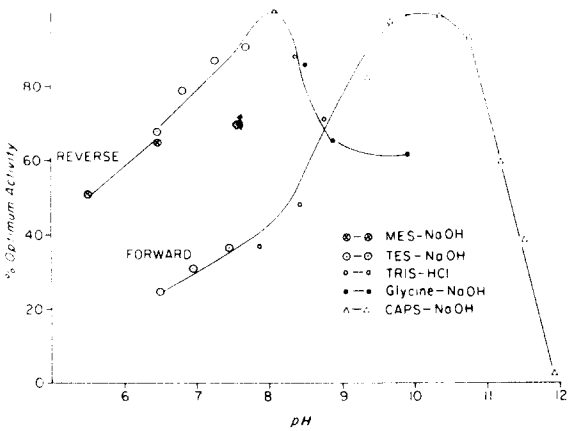

Fig. 7. The effect of pH on the velocity of the forward and reverse reactions. The buffers were as defined in the text. These determinations were made in the presence of saturating ADP (8 mM) with a $MgCl_2$ concentration of 4 mM in the forward direction and in the presence of saturating amounts of both substrates (ATP, 2 mM; AMP, 0.5 mM) in the reverse reaction. The final concentration of each buffer was 0.04 M.

ate kinase activity occurred at pH 10. Even so, all subsequent assays were conducted at pH 8.5. This decision was made based upon the relationship of the pH effects in the forward and reverse reactions as will be discussed below.

The effect of pH upon the velocity of the reverse reaction is also illustrated in Fig. 7. The optimal adenylate kinase activity in this instance occurred at pH 8.0. As the pH changed from 8 to 10, the velocity of the reverse reaction progressively decreased while the velocity of the forward reaction progressively increased. At pH 8.5 the enzyme would be at an intermediate position at which it is most sensitive to variation in intracellular pH. All assays were subsequently conducted in either direction at pH 8.5 since this might well be a position at which the enzyme could be equally as sensitive to changes in the concentratoin of effector molecules or of other physiological parameters.

The effect of substrate concentration on the velocity of the forward reaction

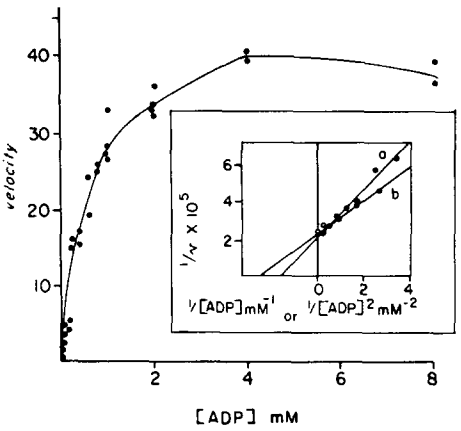

Fig. 8. The effect of ADP concentration on the velocity of the forward reaction. Velocity is given in μmoles ATP produced/min per mg protein. The concentration of Mg^{2+} was varied to be maintained at 1/2 the ADP concentration. Each assay was run in 0.04 M Tris–HCl, pH 8.5, for 30 s. Inset: Line a, 1/[ADP]; Line b, $1/[ADP]^2$.

is illustrated in Fig. 8. The reciprocal Lineweaver–Burk plot is given in the inset. The reciprocal plot of $1/v$ *vs* $1/[ADP]^2$ is also included. Note that both plots are linear over the range plotted and that the x intercept of the $1/[ADP]^2$ plot is equal to the square of the x intercept of the 1/[ADP] plot.

The value for the apparent $K_{m(ADP)}$ by the least squares fitted straight line of the 1/[ADP] reciprocal plot was 0.55 mM. In subsequent assays 4.0 mM ADP was used, to approximate saturating conditions. This concentration is 7 times the K_m and provides 88% saturation[21].

The influence of temperature upon the forward and reverse reactions was also studied. Under the conditions employed the velocity reached a maximum at approximately 57 °C in both directions. The apparent energies of activation under these

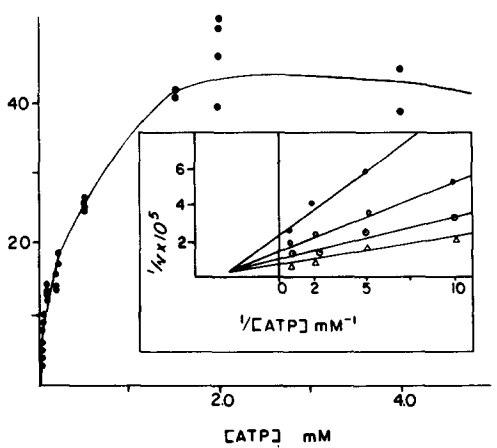

Fig. 9. The effect of ATP concentration on the velocity of the reverse reaction. Velocity is given as μmoles ATP utilized/min per mg protein. The $MgCl_2$ concentration was varied to maintain a constant ratio of 1/2 the ATP concentration. All assays were run in 0.04 M Tris–HCl, pH 8.5, for 30 s. ●—●, 0.05 mM AMP, ○—○, 0.08 mM AMP, ○—○, 0.2 mM AMP, △—△, 0.5 mM AMP.

conditions were calculated from the slopes of the Arrhenius plots (Fig. 5) to be 4.2 kcal/mole per °K in the forward and 4.3 kcal/mole per °K in the reverse reaction.

In the reverse reaction the variation in velocity with ATP concentration was studied and the results are shown in Fig. 9. The reciprocal Lineweaver–Burk plots at various constant concentrations of AMP are given in the inset. The value for the apparent K_m as determined from the secondary plots of these data are 0.11 mM. Since the enzyme exhibited a slight substrate inhibition at 4.0 mM ATP, 2.0 mM ATP ($5 \times K_m$ or 83% saturation) was chosen to approximate saturating conditions to insure freedom from substrate inhibition.

The effect of Mg^{2+} concentration upon the velocity of the reverse reaction is shown in Fig. 6. The Mg^{2+} concentration was varied at three ATP concentrations while AMP was near-saturating (0.5 mM). In the upper curve (ATP saturating, 2 mM) the maximum velocity occurred when the Mg^{2+} concentration was 1.0 mM or 1/2 that of ATP. In the lower curve, (ATP, 0.5 mM) and the lowest curve (ATP, 0.2 mM) the maximum velocity occurred at 1/2 the ATP concentration (0.25 mM and 0.1 mM, respectively).

The variation in velocity with AMP concentration is illustrated in Fig. 10. The reciprocal Lineweaver–Burk plots at various constant concentrations of ATP are given in the inset. The apparent K_m derived from the secondary plots of these data is 0.13 mM.

Due to the pronounced substrate inhibition exhibited at higher concentrations of AMP, 0.5 mM AMP (5 times the K_m or 83% saturating) was subsequently used to approximate saturating conditions and to avoid possible substrate inhibition.

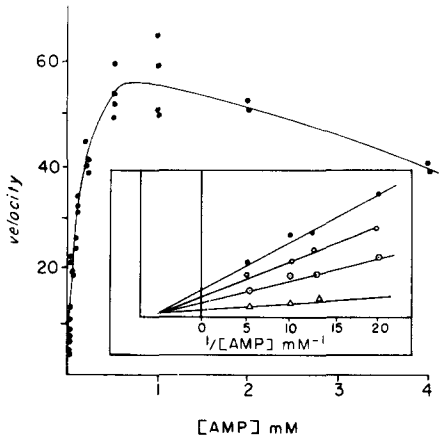

Fig. 10. The effect of AMP concentration on the velocity of the reverse reaction. Velocity is given as μmoles AMP utilized/min per mg protein. The $MgCl_2$ concentration was varied to be maintained at 1/2 the ATP concentration. All assays were run for 30 s in 0.04 M Tris-HCl, pH 8.5. ●—●, 0.1 mM ATP; ○—○, 0.2 mM ATP; ○—○, 0.5 mM ATP; △—△, 2.0 mM ATP.

DISCUSSION

Adenylate kinase from *T. neapolitanus* was purified to near-homogeneity as evidenced by its behavior in disc gel electrophoresis. This preparation, which was

known to be free of any major competing enzymes, was investigated in an effort to clarify the active metabolic role of this enzyme.

Several properties of adenylate kinase from *T. neapolitanus* determined in this study are compared in Table II with those of highly purified enzymes from two other organisms[22–25]. This comparison was made to determine whether adenylate kinase from *T. neapolitanus* possessed any unique properties reflective of its intimate association with this organism's energy metabolism. The enzyme exhibited marked similarity in its isoelectric point and molecular weight to the enzyme from other organisms.

TABLE II

A COMPARISON OF ADENYLATE KINASE FROM *T. neapolitanus* WITH THAT FROM TWO OTHER SOURCES

Property	*T. neapolitanus*	*Rabbit muscle*	*Bovine liver mitochondria*[25]
pI	4.2	4.3 [24]	
Molecular weight	23 500	21 600 [24]	21 500
pH optimum			
Forward	10	8 [23]	7.5
Reverse	8	8 [23]	7.5
Optimal [Mg^{2+}]			
Forward	1/2[ADP]	1/2[ADP] [23]	1/2[ADP]
Reverse	1/2[ATP]	[ATP] [23]	[ATP]
$K_{m\ (ATP)}$ (M)	$3.8 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$ (ref. 28) $3.3 \cdot 10^{-4}$ (ref. 23)	$1.8 \cdot 10^{-3}$
$K_{m\ (AMP)}$ (M)	$1.1 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$ (ref. 23) $5.0 \cdot 10^{-4}$ (ref. 28)	$2.7 \cdot 10^{-3}$
$K_{m\ (ADP)}$ (M)	$5.5 \cdot 10^{-4}$	$1.58 \cdot 10^{-3}$ (ref. 28) $3.3 \cdot 10^{-4}$ (ref. 23)	$1.8 \cdot 10^{-3}$

Likewise the pH optimum determined in the reverse reaction for the enzyme from *T. neapolitanus* was pH 8.0. This is the same as that of rabbit muscle[23] and is only slightly higher than that of the bovine enzyme[25]. In the forward reaction, however, the *T. neapolitanus* enzyme exhibited a pH optimum a full 2 pH units higher than in the other organisms. This characteristic is probably related to the role of this enzyme in the conversion of the end-product of the sulfur oxidation pathway to ATP. Such a relationship is further suggested by the comparable pH optimum of 9.5 for APS reductase of *T. thioparus* when coupled with cytochrome *c* (ref. 26). It is tempting to speculate that there is a close intracellular association between the participants in the sulfur oxidation pathway. Suggestive of this is the apparent protective role which the endogenous cytochrome *c* plays towards adenylate kinase in this organism. As discussed earlier this soluble cytochrome protects adenylate kinase from inactivation during disc gel electrophoresis and during ultrafiltration. It is therefore possible that it extends the same protection *in vivo*.

The pH curves for the 2 reactions of adenylate kinase (Fig. 7) are of further interest regarding the pH optimum. At pH 8.5 the enzyme is in a position where it is extremely sensitive to pH. An increased pH would favor the conversion of ADP to ATP (as well as the formation of APS) and as a result the operation of the sulfur oxidation pathway itself. It is therefore easy to visualize how local fluctuations in pH

in the highly concentrated cytosol could provide the required environment for maximum efficiency in the energy metabolism of this organism.

This function of the enzyme may also be responsible for its atypical optimal ATP:Mg^{2+} ratio. In other organisms[22,25] a 1:1 ratio is observed whereas in *T. neapolitanus* a 1:0.5 ratio is consistently observed. This may be an important regulatory feature for this particular enzyme such that when the Mg^{2+} concentration in the cell is high (ATP concentration low[27]) the conversion of 2 ADP $\rightarrow$ ATP + AMP is favored. Conversely when the Mg^{2+} concentration is low (ATP concentration high) the reverse reaction ATP + AMP $\rightarrow$ 2 ADP would be favored by mass action and the added Mg^{2+} factor. The optimal Mg^{2+}:ADP ratio on the other hand is consistent with that observed in other organisms (1/2[ADP][23,25]). The apparent Michaelis constants derived for the 3 substrates agree well with values reported for the rabbit muscle enzyme[23,28] (other authors have reported values for adenylate kinase from rabbit muscle[29], yeast[30], and human erythrocytes[32]; such values, however, were determined using the coupled enzymatic assay described previously which was found to be entirely inadequate for kinetic investigations as will be discussed in length in a separate publication). The values reported for the bovine liver mitochondrial enzyme[25] are an order of magnitude larger and presumably reflect a distinct role for this enzyme in its mitochondrial milieu. This enzyme is also, in contrast to the rabbit muscle and *T. neapolitanus* enzymes, inactivated by sulfhydryl reducing agents, suggesting it to be a basically different form of the enzyme. The difference in K_m values is therefore less surprising.

In general, the enzyme adenylate kinase from *T. neapolitanus* resembles the enzyme from other organisms. It does exhibit some unique properties however which can be explained in terms of the enzyme's unusual position in the energy metabolism of this organism. The enzyme appears to be highly sensitive to existing physiological conditions, responding sharply to changes in pH, Mg^{2+} concentration or the presence or absence of endogenous cytochrome *c*. Such sensitivity makes regulatory sense as far as the control or maintenance of sulfur oxidation in this organism is concerned. It is a matter for future investigation to determine if other adenylate kinases operating far from equilibrium are subject to the same or comparable sensitivities.

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