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ADENYLATE KINASE FROM BAKER'S YEAST

I. PURIFICATION AND INTRACELLULAR LOCATION

CHE-SHEN CHIU*, SHIRLEY SU** AND PERCY J. RUSSELL
Department of Biochemistry, University of Kansas School of Medicine, Kansas City, Kan. (U.S.A.)

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SUMMARY

1. A purification procedure and some of the general properties of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) from yeast are given.

2. A fraction of yeast adenylate kinase which was immunologically homogeneous was also homogeneous by the following criteria: column chromatography; paper electrophoresis; moving boundary electrophoresis and ultracentrifugal analysis at two pH's. The molecular weight was determined as 41 000.

3. The pH *vs.* activity curves and the K_m of adenylate kinase from yeast are compared with those of myokinase.

4. The subcellular distribution was determined using two methods of cell disruption. Both methods indicate that the adenylate kinase in yeast is not associated with the particulate fractions of yeast.

INTRODUCTION

The adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) from rabbit muscle has been characterized (in this paper it is referred to as myokinase). The catalysis of the reaction 2 ADP = ATP + AMP is known to occur in many microbial, plant and animal tissues. In animal tissues, the enzyme is associated with the mitochondria. The adenylate kinase activity in mitochondrial systems prevents a proper assessment of ATP–ADP exchange reactions due to oxidative phosphorylation enzymes, as well as in other enzyme systems, and so the characteristics of the enzyme is of some interest, particularly in view of the universal occurrence of the activity.

The characterization of adenylate kinases from yeast and tissues other than muscle is not so well established. Adenylate kinase was first detected in yeast by

* Present address: University of Michigan, Department of Biochemistry, Ann Arbor, Mich., U.S.A.
** Present address: University of California, Department of Biophysics and Biochemistry, Davis, Calif., U.S.A.

TRUCCO *et al.*[1]. This paper presents an initial characterization and purification of an ATP:AMP phosphotransferase fraction from baker's yeast, hereafter referred to as yeast adenylate kinase. The adenylate kinase activity from yeast appears to be made up of more than one fraction of protein, which may be separated by electrophoresis and column chromatography. Yeast adenylate kinase has a molecular weight of 41 000 and evidence indicates a lack of free sulfhydryl groups. The pH optima of the forward and reverse reactions are sharply defined and differ from one another. The subcellular distribution of the yeast enzyme indicates no association with any particulate fraction in contrast to adenylate kinase from pigeon skeletal muscle[2], mouse liver[3] and rat liver[4].

The forward direction in this paper is considered as the formation of ATP + AMP from 2 ADP. The formation of 2 ADP from ATP and AMP is called the reverse direction.

METHODS AND MATERIALS

Baker's yeast from the Fleischmann Co. was air dried for 5 to 7 days and then stored at 0–5° until ready to use.

Adenylate kinase in the forward direction was measured by coupling the formation of ATP to the hexokinase (EC 2.7.1.1) reaction plus the glucose-6-phosphate dehydrogenase (EC 1.1.1.48) reaction and measuring the formation of NADPH at 340 mμ by modification of the method of OLIVER[5] at 25°. The reaction mixtures were buffered with 0.033 M potassium phosphate (pH 7.0) unless otherwise stated. Adenylate kinase was measured in the reverse direction by coupling the formation of ADP to the pyruvate kinase (EC 2.7.1.40) reaction *plus* the lactate dehydrogenase (EC 1.1.1.27) reaction and measuring the oxidation of NADH at 340 mμ as in the method of BUCHER AND PFLEIDERER[6]. All enzymes were obtained from the Boehringer Company and all nucleotides were from Pabst Laboratories.

TEAE-cellulose resin was obtained as Cellex T from the Bio-Rad Company and was washed several times with 0.2 M KOH prior to equilibration with the proper potassium phosphate buffer.

Electrophoresis with cellulose acetate paper was performed at room temperature with fresh 0.04 M potassium phosphate (pH 5.6 or 8.4) unless otherwise stated. Protein was detected on paper by staining with Ponceau S[7]. Adenylate kinase activity could be eluted from papers both before and after staining.

Moving-boundary electrophoresis was performed with the Antweiler-designed micro-electrophoresis apparatus[8] made by Boskamp Company under the following conditions: 0.04 M potassium phosphate buffer at pH 5.6 or 8.4, at 25° and with a current of 2 mA.

Ultracentrifugal analyses were carried out with the Beckman Model E centrifuge at 20° with 0.1 M potassium phosphate buffer at pH 5.6 or 6.7 *plus* 0.1 M KCl as the protein medium. The rotation rates were 59 780 rev./min for the sedimentation studies and 11 272 for the approach to equilibrium method of ARCHIBALD[9].

Yeast spheroplasts were prepared by slight modification of the method of DUELL, INOUE AND UTTER[10]. Spheroplast formation was followed with a phase contrast microscope and incubation of prepared yeast cells with snail enzyme (an enzyme mixture called Glusulase from Endo Laboratories) was usually about 1.5 h at

which time at least 95% cell lysis could be obtained. Separation of various subcellular fractions was accomplished by a modification of the differential centrifugation method of SCHNEIDER AND HOGEBOOM[11]. All sedimenting fractions were washed twice and all calculations were based on 10 g of yeast.

Disruption of the yeast by use of the Braun shaker[12] was under the following conditions: 30 g of fine glass beads; 30 ml of 0.25 M sorbitol containing 8 g of yeast; and shaking periods of 10 sec. The 10-sec period yielded 50% cell breakage as determined by the total protein obtainable by extended shaking periods. All calculations were based on 100% disruption of 10 g of yeast for the purpose of comparison with the method above.

Protein concentrations were determined by the spectroscopic method of WARBURG AND CHRISTIAN[13], unless stated otherwise.

EXPERIMENTAL

Purification of enzyme

After ammonium sulfate precipitation, 15 min was allowed prior to centrifugation for all precipitate collections, and all centrifugations were at 12 000 × *g* for 15 min. All operations were carried out at 0–5° unless otherwise stated.

Step 1. To each 1800 ml of 0.17 M ammonium monohydrogen phosphate, 600 g of dried baker's yeast was added and stirred for 4 to 5 h at 37°. After centrifugation, the protein supernatant fluid concentration was adjusted to 40 to 50 mg/ml with water.

Step 2. An equal volume of acetone was added slowly to the supernatant with stirring and the temperature was gradually lowered to −15°. The 50% acetone solution was stirred for 15 min and centrifuged at −15°. After centrifugation, acetone was completely removed from the supernatant fluid in a Rinco Rotatory flash evaporator *in vacuo*, with the flask containing the acetone solution immersed in a 30° bath. The acetone-free solution was then made 65% saturated by the addition of solid ammonium sulfate. The collected precipitate may be stored at −20° for several days with little loss of activity.

Step 3. The precipitate was then suspended in 23 ml of 20% saturated ammonium sulfate previously adjusted to pH 7 with ammonium hydroxide and stirred for 15 min. The insoluble material was removed. The supernatant fluid had a protein concentration of about 20 mg/ml which was precipitated by the addition of solid ammonium sulfate to 50% saturation. The collected precipitate was then taken up with enough water to give a protein solution containing 30 mg/ml.

Step 4. About 0.12 vol. of 1 M trichloroacetic acid was added slowly to the protein solution with stirring. The precipitate was centrifuged immediately and then washed once by suspension in 15 ml of 0.01 M potassium phosphate buffer, (pH 7.0) to remove inert protein. The washed precipitate was collected by centrifugation and then extracted by suspension in 12 ml of 0.1 M potassium phosphate buffer (pH 7.0). The insoluble material was removed and the supernatant fluid was then brought to 65% saturation by the addition of solid ammonium sulfate. The collected precipitate was dissolved in enough water to give a final protein concentration of 15 to 20 mg/ml.

Step 5. The protein solution was then brought to pH 3.5–4.0 by the dropwise addition of 3 M HCl and rapid stirring. The suspension was then stirred for 5 min;

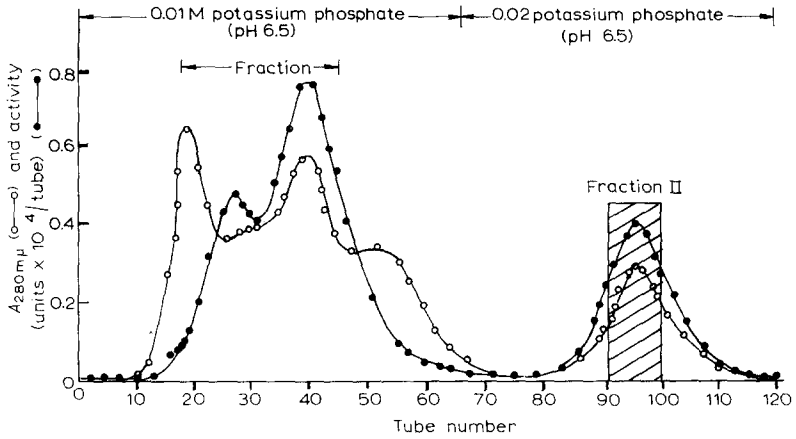

Fig. 1. Chromatographic separation of yeast adenylate kinase, Fraction II, on TEAE-cellulose. The open circles (○—○) refer to protein absorbances and the closed circles (●—●) correspond to total activities in 6 ml of eluate. The eluants are indicated and other conditions are in the text.

inert protein was removed; and the supernatant fluid was neutralized by the dropwise addition of 3 M KOH. The adenylate kinase activity was precipitated by bringing the neutralized solution to 65% saturated ammonium sulfate by the addition of the solid. The collected precipitate was then dissolved in about 1.5 ml of water and the solution was dialyzed against distilled water overnight to remove all salt for Step 6. The dialysis results in little or no loss of activity and the precautions required for the dialysis of myokinase[14] are not necessary. The dialyzed solution may be stored in the frozen state for extended periods without loss of activity.

Step 6. About 300 mg of dialyzed protein in 12 to 15 ml of solution was applied to a 2 cm × 42 cm column of TEAE-cellulose resin equilibrated with 0.01 M potassium phosphate buffer (pH 6.5). All eluting buffers were at pH 6.5 and were delivered at a rate of 24 ml/h, unless stated otherwise. As shown in Fig. 1, two peaks of activity are discernible by elution with 0.01 M potassium phosphate. The studies of this paper have been limited to fractions designated as Fraction II in Fig. 1. Only those portions of the activity peak yielding a constant specific activity were collected, pooled and further studied. Whenever Fraction II was contaminated, by reason of a variation in

TABLE I

PURIFICATION OF AN ADENYLATE KINASE FRACTION FROM BAKER'S YEAST

Step	*Fraction*	*Total units of activity*	*Specific activity*	*% recovery*
1	Crude autolysate	62 000	1.2	100
2	Acetone soluble	37 000	45	60
3	Ammonium sulfate extract	35 000	68	56
4	Extract of trichloroacetic acid precipitate	17 500	175	28
5	pH 4-soluble	16 000	540	26
6	Fraction II	4 350	1900	7

the protein pattern or specific activity, it was then pooled and the complete chromatography procedure was repeated.

A summary of the purification procedure and the yields is given in Table I. All protein concentrations given in the procedure above were critical, and deviation outside of given limits resulted in a marked decrease in either specific activity or total activity of both.

Criteria for purity

The apparent association of adenylate kinase activity with more than one protein fraction would not appear to be an artifact of the column chromatography procedures since three major bands of activity can also be detected by paper electrophoresis of proteins from Step 5. After the collection of Fraction II, the following criteria for purity for that fraction were established; by gel diffusion, a single precipitation band was obtained with Fraction II against anti-sera from guinea pig and rabbit for proteins in Step 5 of the purification; a single and coincident band of protein and activity was obtained by paper electrophoresis at pH's 5.6 and 8.4; a single protein peak was obtained by moving-boundary electrophoresis at pH's 5.6 and 8.4; and similarly a single peak was obtained by ultracentrifugal analysis at two pH's.

Molecular weight determinations

The molecular weight of yeast adenylate kinase (Fraction II) was determined by the approach to equilibrium method of ARCHIBALD[9] and the sedimentation velocity method[14]. Both methods involved an assumption of the partial specific volume as 0.74 and the latter method a calculation of the apparent diffusion coefficient, D_{app}.[15]. The constants determined by these methods and the agreement obtained for the molecular weight determinations are given in Table II.

TABLE II

ULTRACENTRIFUGAL ANALYSIS OF YEAST ADENYLATE KINASE, FRACTION II

Determination	*Method*	*Value*
Molecular weight	Sedimentation velocity[14]	39 800
Molecular weight	ARCHIBALD[9]	41 200 ± 700*
Sedimentation constant, s_0	—	2.96 S**
Apparent diffusion coefficient, $D_{app.}$	Graphic[14]	$7 \cdot 10^{-7}$ cm²/sec**

* This standard error of mean was calculated from both the liquid–air and liquid–silicone interfaces.

** This value was not corrected for the salt which was 0.10 M potassium phosphate buffer (pH 6.7) *plus* 0.10 M KCl.

Activity vs. pH

As shown in Fig. 2, the optimum for the forward reaction is 7.7 while the reverse reaction optimum is 7.2 in phosphate buffer. The sharpness of the optima obtained with yeast adenylate kinase is in contrast to the broad optima of myokinase[16]. The reverse reaction rate was greater than the forward reaction rate for both myokinase[16] and yeast adenylate kinase, with both yielding a ratio of 1.6 for the reverse rate/forward rate, if the optimum pH rates of the yeast enzyme for each direction are used.

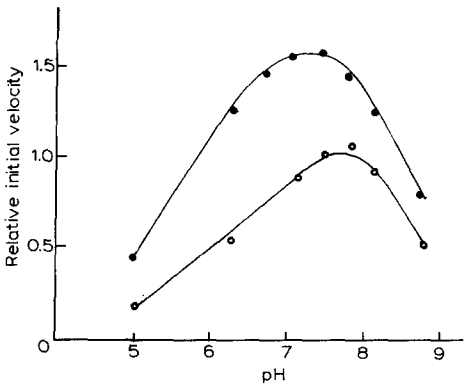

Fig. 2. Effect of pH on the rate of the reverse reaction (●—●) and forward reaction (○—○). Assay conditions are in the text.

K_m determinations

The K_m for ADP was determined at pH 7.0 and 24° between the concentrations of $5 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ M. The K_m value obtained was $2.7 \cdot 10^{-4}$ M and did not vary between pH 6.0 and 7.3. This value compares with the $3 \cdot 10^{-4}$ K_i values for ADP determined with muscle adenylate kinase at pH 8 and 25° by NODA[16] and $16 \cdot 10^{-4}$ M at 30° and pH 7.5 as determined by CALLAGAN AND WEBER[14]. A LINEWEAVER AND BURK[17] plot for the K_m determination is given in Fig. 3. It should be noted that a linear plot is obtained for 1/ADP as the abcissa. NODA[16] obtained a linear plot with $(1/ADP)^2$ as would be expected from the equation of the reaction, 2 ADP ⇌ ATP + AMP. The linearity or non-linearity of the plot of 1/ADP for this reaction can be shown to depend upon the concentration range used for the graphic presentation. A squared term is present in the kinetic equation, but it becomes less significant at the higher substrate concentrations[18]. A more detailed account of these changes and other kinetic properties are presently under study. It may also be noted that ADP exhibits a pronounced substrate inhibition effect.

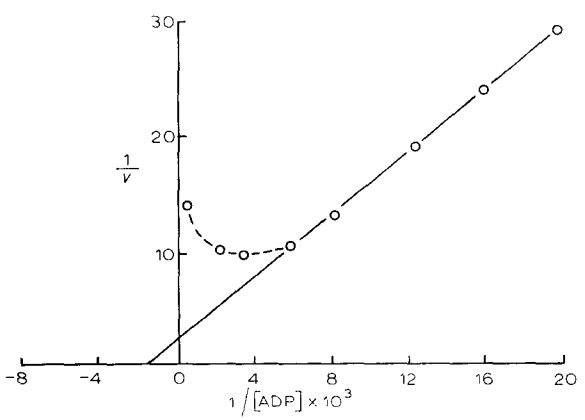

Fig. 3. Determination of the K_m of ADP for the forward reaction. The conditions for measurement are in the text.

Effect of sulfhydryl reacting reagents

Since it had been demonstrated that myokinase required two –SH groups for activity and was therefore inactivated by sulfhydryl reagents[19], it was of interest to examine the effect of various sulfhydryl reacting reagents on yeast adenylate kinase. Table III shows that five sulfhydryl reagents had little or no inhibitory effect on yeast adenylate kinase activity after prolonged incubation at 25°. Two of the reagents, mercuric chloride and silver nitrate gave rise to precipitates, which would indicate the denaturing effect of these reagents. Restoration of the activity losses by incubation with cysteine or glutathione were negative. Attempts were also made to determine the presence of sulfhydryl groups with the reagent of ELLMAN[20], 5,5'-dithiobis-(2-nitrobenzoic acid). Using a molar absorbance index of 13 600 for the reagent of ELLMAN, 19.8 mμmoles of –SH groups were detected per 67 mμmoles of yeast adenylate kinase and are considered significant.

Intracellular distribution

It was of interest to determine the intracellular distribution of the yeast adenylate kinase for comparison with animal systems, and particularly in view of the separa-

TABLE III

LACK OF INACTIVATION OF YEAST ADENYLATE KINASE ACTIVITY BY SULFHYDRYL REACTING REAGENTS

Each 0.5-ml solution contained 0.64 mg of Fraction II, 25 mM potassium phosphate (pH 7.0) and 1 mM sulfhydryl reagent and the mixture was incubated for the periods of time indicated. The activity remaining was determined on a 20 000-fold diluted portion of the incubation mixtures by measuring the rate of the reverse reaction. The percent activity remaining was based up the appropriate time control, which contained no sulfhydryl-reacting reagent.

Sulfhydryl reagent (0.5 mM)	*Incubation time (h)*	*Percent activity remaining*
None	0	100
	0.5	100
	6	100
	20	85
N-Ethylmaleimide	0.5	102
	6	100
	20	80
Idoacetic acid	0.5	102
	6	100
	20	85
p-Hydroxymercuribenzoate chloride	0.5	93
	6	93
	20	85
Mercuric chloride	0.5	67*
	6	63*
	20	59*
Silver nitrate	0.5	100
	6	95**
	20	83**

* Heavy precipitation observed.
** Slight turbidity observed.

TABLE IV

ADENYLATE KINASE ACTIVITY IN SUBCELLULAR FRACTIONS

Protein determinations were by the biuret method[24] and correction was made for the protein present as snail enzyme. All calculations are based on 10 g of yeast.

Method of cell rupture	*Subcellular fraction*	*Total protein (mg)*	*Units of activity**	*Specific activity*
Braun shaker	mitochondria	42	5.5	0.13
	microsomes	82	4.9	0.06
	supernatant	257	402	1.56
	total	381	412	1.08
Spheroplast Lysis	mitochondria	44	5.0	0.11
	microsomes	63	6.5	0.10
	supernatant	276	480	1.74
	total	383	492	1.28

* Each value was corrected for the small endogenous NADH oxidase and ATPase activities of each fraction.

tion of more than one adenylate kinase fraction in yeast. To simplify the number of manipulations for the separation and testing of various subcellular components, it was ascertained that the snail enzyme used for spheroplast formation in no way interfered with the assay for adenylate kinase.

Table IV shows a comparison of the distribution of adenylate kinase among subcellular fractions by two methods of cell disruption. Cell fragments and debris are excluded from Table III and constituted approximately 10% of the total adenylate kinase activity after being washed twice. The distribution of adenylate kinase among the subcellular fractions obtained by the two methods is strikingly similar. It can be seen that not only was the amount of adenylate kinase low in the microsomal and mitochondrial fractions, but also the specific activities in these fractions were one-tenth of that in the supernatant fraction.

DISCUSSION

Adenylate kinase from yeast and the homologous enzyme from muscle show some interesting contrasts. The pH activity curves are sharp and well defined in the case of yeast adenylate kinase and broad in the case of myokinase[16]. Subcellular distribution again marks a contrast since in animal cells[2-4] adenylate kinase has been associated with the mitochondria of the cell, although the heart muscle appears to contain appreciable quantities of the enzyme in the supernatant fluid fraction[22]. Adenylate kinase has been so associated with mitochondria in animal cells that the enzyme has been suggested to function as an adjunct to oxidative phosphorylation processes[23]. The distribution of adenylate kinase in yeast, by contrast, was such that the enzyme appears to be cytoplasmic in origin, which accounted for greater than 90% of the total cellular activity.

Of particular interest was the recalcitrance of yeast adenylate kinase to sulfhydryl reagents. All evidence indicates that there are no freely reactive –SH groups in the yeast enzyme. By contrast, myokinase has been shown to possess two free sulfhydryl groups, which are required for activity, as evidenced by the loss of activity, upon

reaction with sulfhydryl reagents[2]. It was the view of MAHOWALD, NOLTMANN AND KUBY[19] that the "stoichiometric inhibition" of myokinase with sulfhydryl reagents was evidence for the participation of the -SH groups at the active site. If the effects of adducts to proteins may be viewed in a broader light of allosteric effects, it then becomes presently difficult, if not impossible, to distinguish between an adduct which causes an inhibition by a stoichiometric allosteric alteration and an adduct which involves a specific interaction at the active site. Suffice to say that myokinase requires free -SH groups for a full expression of enzymatic activity and that homologous enzymes from yeast and beef liver[21] apparently do not. Preliminary amino acid analyses indicated that the yeast enzyme contained two half-cysteine residues per mole. This means that the -SH groups were either buried, as indicated by the lack of reactivity with the reagent of ELLMAN[20], or were present as disulfide, as apparently was the case with the homologous enzyme from beef liver[21]. Since there are such wide variations in the sulfhydryl requirements among the homologous adenylate kinases from various sources, and since they all do catalyze the same reaction with high specificity, it seems more reasonable to consider that the sulfhydryl requirements of these enzymes are related to a more gross structural features than the active site. If this is not the case then marked structural differences must exist at the active sites of the various homologous enzymes. Studies concerning the properties of the active sites are presented in the following paper.

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