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ADENYLATE KINASE FROM BAKER'S YEAST

II. SUBSTRATE SPECIFICITY

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SUMMARY

1. K_m's were determined for nucleotides serving as substrates for yeast adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), which included AMP, ADP, ATP, ITP, GMP, and GTP. IMP could not serve as a substrate and ITP greatly influenced and increased the K_m of AMP. The adenosine deoxyribose derivatives could serve as substrates but at one thousandth the rate of the ribose homologs.

2. The specificity of yeast adenylate kinase was determined with respect to the requirements for the ribose and purine moieties and the substituents on the purine ring. Among the adenosine nucleotides, the decreasing order of specificity was determined as follows: ADP>AMP>ATP.

3. Several divalent cations were effective in potentiating the enzymic activity. The order of decreasing effectiveness was $Mg^{2+}>Ca^{2+}>Mn^{2+}>Ba^{2+}$.

4. Binding studies indicated that yeast adenylate kinase has two binding sites with different binding specificities. Evidence is presented to show that the site binding nucleotides uncomplexed with cation has the higher specificity.

5. Comparisons are made between yeast adenylate kinase and myokinase with respect to similarities and differences.

INTRODUCTION

In a previous paper[1], an ATP:AMP phosphotransferase (EC 2.7.4.3) from baker's yeast was purified and partially characterized. The present paper is a further extension of the characterization of adenylate kinase from yeast.

Emphasis in this paper was the substrate specificity of the yeast enzyme, which not only included the purine and pyrimidine nucleotides, but also the metal requirements and some binding studies with the adenosine nucleotides. The K_m values of all

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of the nucleotides which served as substrate for yeast adenylate kinase were determined.

These studies showed that the adenylate kinase from yeast was specific for the purine nucleotides since no pyrimidine nucleotide served as a substrate under any of the conditions tested. Yeast adenylate kinase also possessed an almost absolute specificity for ADP since no other nucleotide diphosphate tested could serve as a substrate at low enzyme concentrations. The deoxy derivatives of the adenosine nucleotides were also studied with high concentrations of the enzyme and they were found to behave similar to the ribose derivatives.

MATERIAL AND METHODS

The following materials were used in the experiments.

Enzymes. Yeast adenylate kinase was purified from baker's yeast as described by CHIU, SU AND RUSSELL[1]. The following enzymes were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.): pyruvate kinase (EC 2.7.1.40), Type II; lactate dehydrogenase (EC 1.1.1.27), Type II, crystalline; hexokinase (EC 2.7.1.1), crystalline; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), Type V; and 5'-AMP deaminase (EC 3.5.4.6).

Chemicals and materials. NADH and the nucleotide phosphate derivatives were obtained from the Pabst Chemical Company (St. Louis, Mo., U.S.A.) as the sodium salt, except for dADP which was obtained as the sodium salt from the Sigma Chemical Co. DEAE-cellulose for the thin-layer chromatography was obtained from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) as Cellex D for thin-layer chromatography. Sephadex G-25, medium was a product of Pharmacia (Uppsala, Sweden).

Measurement of the enzymic activity of adenylate kinase. Several methods for the measurement of the activity of yeast adenylate kinase were employed. If not specified, the measurement of the reverse and forward reactions were by the methods BÜCHER AND PFLEIDERER[2] and OLIVER[3]. Whenever the cation became the limiting factor (as in studies of the cation specificity and the effects of magnesium ion concentrations), then the method of NIKIFORUK AND COLOWICK[4] was employed. This latter method depended upon the measurement of the AMP formed in the case of the forward reaction and the amount of AMP decreased in the case of the reverse reaction. In practice, 1.0-ml portions of reaction mixtures were removed at appropriate times, usually 1 min, and the reactions were stopped by addition to 1.95 ml of 0.01 M sodium citrate buffer and 0.05 M EDTA at pH 6.5. The citrate–EDTA terminated reactions were then measured for the content of AMP. The measurements of the initial rates of the conversion of 5'-AMP to 5'-IMP were initiated by the addition of 0.02 ml of 5'-AMP deaminase (2000 units/ml) to the reaction-terminated mixtures or to AMP standard solutions, which also contained the appropriate amount of yeast adenylate kinase. This assay shall be referred to as the deaminase assay hereafter.

Since the measurement of the reactions of yeast adenylate kinase reported in this paper required the use of two auxillary enzyme systems, the deaminase system and the method of OLIVER[3] were compared with a thin-layer chromatographic method which was independent of any additional enzyme system. For the purpose of comparison, the K_m value of ADP was determined using the three methods under comparable reaction conditions. The thin-layer chromatographic method was that described

by RANDERATH[5] using DEAE-cellulose plates and 0.02 M HCl to develop the plates in 60 min. It was ascertained that the addition of 10 μl of reaction mixture to the plates caused an immediate termination of the reaction. After development of the plates, the nucleotide spots were located with an ultraviolet lamp, the spots were scraped off of the plates with a scalpel and then quantitatively transferred to 1.5 ml of 0.1 M HCl. The AMP, ADP and ATP so obtained were determined spectrophotometrically, based upon the method of VISCHER AND CHARGAFF[6]. The results of the three K_m value determinations for ADP were as follows: the thin-layer chromatographic method yielded value of 0.36 mM; the deaminase method yielded a value of 0.33 mM; and method of OLIVER[3] yielded a value of 0.27 mM. The agreement among the three methods was considered good.

The binding studies employed the method of HUMMEL AND DREYER[7], which was analogous in principle to equilibrium dialysis. A 100 cm long $\times$ 0.4 cm in diameter column of Sephadex G-25 was equilibrated with 50 mM Tris and approximately 50 μM nucleotide at pH 8.0. Approximately 4 mg of yeast adenylate kinase in 1 ml of the solution used to equilibrate the column was placed on column. The column was then eluted with the equilibration solution. Fractions of about 1 ml were collected for about 30 min. Each fraction was read at 260 mμ and 280 mμ, and absorbance at 260 mμ, was plotted against the volume of the eluate. The total amount of nucleotide bound to the enzyme was determined from the plot with a planimeter by measuring the area falling below the base line of the eluting medium (see Fig. 2).

TABLE I

EFFECT OF DIVALENT CATIONS ON YEAST ADENYLATE KINASE ACTIVITY

All concentrations of the chloride salts were 0.1 mM. The reaction rates were measured by the adenylate deaminase assay method given in the text, which was unaffected by the salt concentration employed.

	Divalent cation			
	Mg^{2+}	Ca^{2+}	Mn^{2+}	Ba^{2+}
Relative reaction rate	100	80	60	15

RESULTS

Metal ion requirements

Yeast adenylate kinase required a divalent metal for activity since incubation of the enzyme with the substrates ATP *plus* AMP or ADP without a divalent metal did not result in formation of any detectable quantities of products by paper or thin-layer chromatographic techniques. As shown in Table I, the specificity of the divalent cation required for the expression of enzyme activity was rather broad.

It was interesting to note that $CaCl_2$ catalyzed the yeast adenylate kinase reaction. This observation was in contrast to myokinase where there appeared to be some doubt about the utility of the calcium ion (see BOWEN AND KERWIN[8] and contrast NODA[9]). It may also be noted that $CaCl_2$ expressed a greater relative activity than did $MnCl_2$ with the adenylate kinase from yeast and that some activation with $BaCl_2$ was observed (contrast NODA[9]).

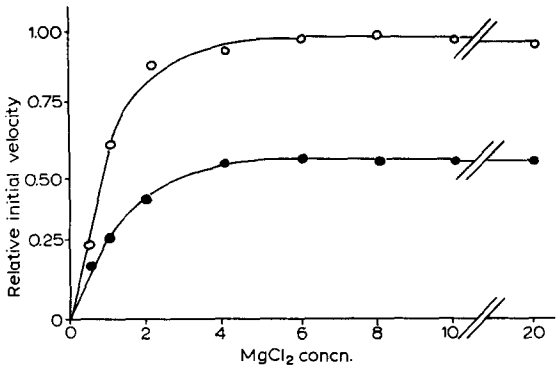

Fig. 1. Effect of magnesium ion concentration on yeast adenylate kinase activity. For the forward direction, indicated by closed circles (●—●), the concentration of ADP was 1.0 mM. For the reverse reaction, indicated by open circles (○—○), the concentration of ATP and AMP were both 0.5 mM. The reactions were carried out at 25° in 30 mM potassium phosphate buffer at pH 7.8 for the forward reaction and pH 7.0 for the reverse reaction. The reaction rates were determined by the thin-layer chromatographic method.

Effect of magnesium ion concentration

Fig. 1 shows that magnesium ion had no inhibitory effect on the yeast adenylate kinase reaction. Moreover, the maximum enzymic activity was reached when the ratio of the magnesium to total nucleotide was about two, both for the forward and the reverse reactions. NODA[9] observed a sharp maximum activity with myokinase when the ratio of magnesium ion to ATP was one in the reverse reaction and again when the ratio of magnesium to ADP was one-half for the forward reaction with myokinase. When the concentration of the magnesium exceeded the total nucleotide by a factor of two or greater the initial velocities of the reaction were decreased to about one-half of the maximum rate obtained[9].

Substrate specificity

Yeast adenylate kinase appeared specific for the adenosine nucleotide derivatives, but could react with the purine nucleotides of guanosine and inosine in certain combinations with an appropriate adenosine nucleotide. Under no condition tested could any of the nucleotides of cytidine or uridine serve as a substrate as determined by paper chromatography analyses for products after prolonged incubations.

The only nucleotide diphosphate which could serve as a substrate was ADP. A very low rate of activity was detected with dADP and measurable rates of activity could be obtained by increasing the enzyme concentration about 1000 times greater than that used for rate measurements using ADP.

Table II gives the relative initial rates of reaction of all of the nucleotides that could serve as substrates for yeast adenylate kinase. The forward reaction of the enzyme at pH 7.0 was the basis for comparison. In addition, the K_m values of all of the nucleotides which underwent catalysis by yeast adenylate kinase are given also. Several interesting features were revealed.

It can be seen that substitution of AMP by another purine mononucleotide caused a greater decrease in the rate of the reverse reaction than a corresponding substitution of another trinucleotide for ATP. This greater dependence on AMP for a

TABLE II

SUBSTRATE SPECIFICITY OF YEAST ADENYLATE KINASE: RELATIVE REACTION RATES AND K_m VALUES AT pH 7

Assay conditions are given in the text.

Reaction	*Relative rate*	*K_m value (M)*
2 ADP → AMP + ATP	100	$K_{ADP} = 2.7 \cdot 10^{-4}$
AMP + ATP → 2 ADP	214	$K_{AMP} = 5.8 \cdot 10^{-5}$ $K_{ATP} = 5.4 \cdot 10^{-5}$
AMP + dATP → ADP + dADP	135	$K_{AMP} = 1.7 \cdot 10^{-4}$ $K_{dATP} = 4.0 \cdot 10^{-4}$
dAMP + ATP → ADP + dADP	46	$K_{dAMP} = 4.0 \cdot 10^{-4}$ $K_{ATP} = 1.0 \cdot 10^{-4}$
AMP + GTP → ADP + GDP	22	$K_{AMP} = 1.1 \cdot 10^{-4}$ $K_{GTP} = 1.0 \cdot 10^{-4}$
GMP + ATP → ADP + GDP	19	$K_{GMP} = 4.0 \cdot 10^{-5}$ $K_{ATP} = 1.1 \cdot 10^{-4}$
AMP + ITP → ADP + IDP	10	$K_{AMP} = 2.0 \cdot 10^{-3}$ $K_{ITP} = 3.1 \cdot 10^{-4}$
dAMP + dATP → 2 dADP	0.53	$K_{dAMP} = 1.8 \cdot 10^{-4}$ $K_{dATP} = 9.1 \cdot 10^{-5}$
2 dADP → dAMP + dATP	0.13	$K_{dADP} = 2.0 \cdot 10^{-3}$

higher catalytic rate reached an extreme with the inosine nucleotides, since IMP could not serve as a substrate while ITP could. It may also be seen that the 2′-hydroxyl group of the ribose was needed for maximum activity since substitution by one 2′-deoxy derivative of the adenosine nucleotides resulted in a marked decrease of the initial reaction rate. An even greater decrease in the initial reaction rates was observed when the adenine moiety was substituted, as indicated by the greatly decreased rates with the guanosine and inosine nucleotides.

With respect to the K_m values given in Table II, in general, it appeared that the substitution of other mono- or trinucleotide for AMP and ATP, respectively, did not effect the K_m value of the remaining adenosine nucleotide by a factor greater than three, except in one notable instance. The substitution of ITP for ATP in the reverse reaction caused an increase of 300-fold in the K_m value of AMP compared to the K_m when ATP was used. This was of particular interest since it was already mentioned that IMP could not serve as a substrate.

It was also of interest that the K_m values of the dATP and dAMP were in the same order of magnitude as the values obtained for ATP and AMP, even when both substitutions were made simultaneously. On the other hand, the K_m value of dADP was greater than that of ADP by one order of magnitude.

Binding studies

The binding of the adenosine nucleotides to yeast adenylate kinase was studied using the method of HUMMEL AND DREYER[7]. The results of these studies are shown in Fig. 2. The nucleotide concentrations used for these studies were of the same order of

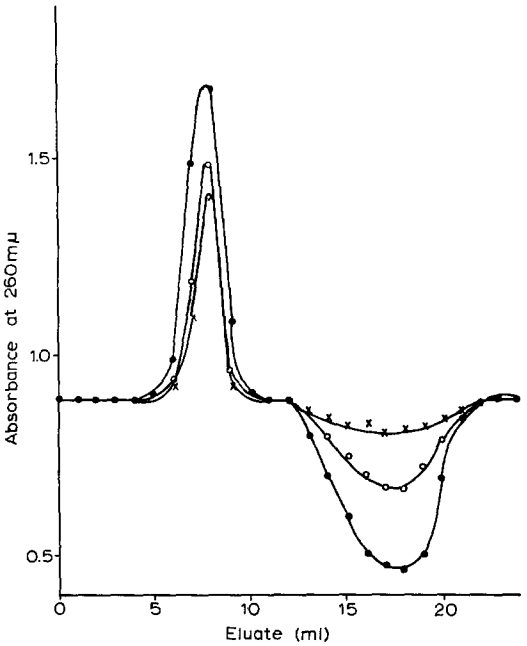

Fig. 2. The figures given represent a composite of five separate experiments with the scales proportionally adjusted to permit a direct comparison among them. The details are given in Table III. The concentration of the nucleotides in the solutions used to equilibrate and elute the columns were as follows: 50 μM ADP; 60 μM ATP; and 53 μM AMP. Where indicated the concentration of magnesium chloride was 125 μM. The closed circles (●—●) correspond to ATP and ADP without magnesium chloride. The open circles (○—○) correspond to ATP *plus* magnesium chloride. The crosses (×—×) correspond to AMP with and without magnesium chloride.

magnitude as the K_m values for AMP and ATP. Fig. 2 shows that the AMP, ADP and ATP were not all bound by the enzyme to the same extent under similar conditions. The presence of magnesium ion decreased the binding capacity of the enzyme for ATP. On the other hand, AMP was not affected by the presence or absence of magne-

TABLE III

BINDING STUDIES OF ADENOSINE NUCLEOTIDES TO YEAST ADENYLATE KINASE

The concentration of the nucleotides in the solutions used to equilibrate and elute the Sephadex G-25 columns were as follows: 50 μM ADP; 60 μM ATP; and 53 μM AMP. Where indicated, the concentration of magnesium ion as the chloride was 125 μM. Other conditions are given in the text.

Nucleotide	*Nucleotide bound (μmoles)*	*Enzyme (μmoles)*	*Moles of nucleotide* / *Moles of enzyme*
ADP	0.162	0.069	2.3
ATP	0.197	0.085	2.3
ATP, Mg^{2+}	0.102	0.085	1.2
AMP	0.022	0.069	0.3
AMP, Mg^{2+}	0.041	0.092	0.4

sium ion and was bound to a considerably less extent than ATP under either condition. The binding of ADP to the enzyme was examined only in the absence of magnesium ion to avoid catalysis, and was the same as ATP under that condition.

Table III gives the quantitative details of the elution patterns shown in Fig. 2. The molar concentration of yeast adenylate kinase was based upon a molecular weight of 42 000 as determined by CHIU, SU AND RUSSELL[1]. The molar ratios of nucleotide bound to enzyme would indicate that less than 1 mole of AMP was bound, 1 mole of the magnesium–ATP complex was bound; and 2 moles of ADP and ATP in the absence of magnesium ion were bound.

DISCUSSION

Differences between adenylate kinase from yeast and muscle were again as evident in these studies as they were in those previous[1]. Among the outstanding differences were substrate specificity, the specificity of the divalent cation required for catalysis and the effect of the magnesium ions on the binding of the substrate. Other differences have also been noted.

Adenylate kinase from rabbit muscle has shown a high degree of specificity for the nucleotide diphosphate since only ADP and CDP were found to serve as substrates[9]. Adenylate kinase from yeast, on the other hand, utilized only ADP as a substrate and was inactive against all other nucleotide diphosphates under the usual conditions for detecting catalysis. Activity of the enzyme with dADP could be detected, but it was of the order of one-thousandth of that with ADP. The substitution of dAMP or dATP for the corresponding ribose derivatives could catalyze the reaction with the usual enzyme concentrations but with a diminished initial reaction rate. The specificity of the yeast enzyme for the adenosine nucleotides was not absolute however. In lieu of ATP, both GTP and ITP could substitute in the reverse reaction. Of the mononucleotides, only GMP was a permissible substitution for AMP in the reverse reaction. The requirement of yeast adenylate kinase for a 6-aminopurine moiety did not appear to be as rigid as that cited for myokinase[10].

It was interesting to note that the K_m values of all the ribose nucleotides which serve as substrates were within an order of magnitude of one another with one notable exception. The substitution of ITP for ATP increased the K_m of AMP by more than two orders of magnitude, while a similar substitution by GTP caused about a 2-fold increase in the K_m value of AMP. Since it was IMP that could not serve as a substrate, this engendered speculation about the structural requirements for substrate binding in which the hypoxanthine moiety represented at best a poor fit at the active site of the yeast enzyme. This was further supported by the fact that the substitution by ITP caused the greatest decrease in reaction rate as result of a single substitution for an adenosine nucleotide. The greatly diminished activity observed when both nucleotides in the reaction were deoxy derivatives also indicates the 2′-hydroxyl group of the has some importance in either the binding or the rate of catalysis. In either case, the diminution of activity was greatest when dADP was substituted for ADP.

From the above, the specificity of yeast adenylate kinase has evolved. In decreasing order, the specificity of the adenosine nucleotides was ADP>AMP>ATP. The 2′-hydroxyl position of ribose appeared to be of less influence than the adenosine

moiety and the absence of an amino group on the purine ring greatly diminished the role of the purine nucleotide as a substrate.

The divalent cation requirements of yeast adenylate kinase were less restrictive than those for myokinase. Calcium ion did not appear to be able to potentiate the myokinase reaction[9]. Yeast adenylate kinase, to the contrary, not only utilized calcium ion, but resulted in a greater activity than the manganese ion. In addition, the barium ion was also able to potentiate catalysis, but at a greatly reduced rate. A pronounced inhibition of the initial reaction rates of myokinase was noted when the magnesium ion concentrations were in excess of the nucleotide concentrations[9]. Such inhibitory effects were absent with the yeast enzyme since a 20-fold excess ratio of magnesium ion to nucleotides ATP and ADP showed no significant decrease in the rate of catalysis. Though not shown here, no inhibition by phosphate buffer was in evidence with the yeast enzyme, while the same buffer caused about a 30% decrease in reaction rate of myokinase compared with Tris[9].

Of particular interest is the comparison of the effect of magnesium on the binding of the adenosine nucleotides to the enzymes. KUBY, MAHOWALD AND NOLTMANN[11] showed that about 2 moles of AMP, ADP, ATP and Mg–ATP were bound per mole of myokinase. No effect of magnesium ion on the binding of the substrates was observed. The binding of ATP to yeast adenylate kinase was, on the other hand, profoundly affected by the presence of magnesium. The presence of magnesium ion clearly decreased the ATP bound to yeast adenylate kinase by one-half. In the absence of magnesium ion the amount of ATP and ADP bound was equal. AMP was bound to the least extent and appeared to be unaffected by magnesium ion. A reasonable postulation, in the light of these findings was that the two binding sites on the yeast adenylate kinase have different specificities. The differences with respect to the binding characteristics would be envisioned as follows. In the absence of magnesium ions, ATP and ADP may bind to two binding sites. In the presence of magnesium ions, Mg–ATP and Mg–ADP are restricted to the one site for the metal complexed nucleotides. Presumably then, the site that binds AMP and ADP as uncomplexed nucleotides is the more specific. This interpretation is in agreement with that suggested by NODA[9,10] which was based on the effect of the substrates on the kinetics of myokinase.

The low extent of binding of AMP by yeast adenylate kinase is not understood at this time, but there is other indirect evidence to indicate that the relationship between AMP and the enzyme is not so firm as that between ATP and ADP. Recent studies in our laboratories with anti-serum made against yeast adenylate kinase showed that ATP, Mg–ATP and ADP prevented inactivation of the enzyme by the anti-serum to a much greater extent than AMP.

In conclusion, the contrast between the adenylate kinases from yeast and muscle have been further established. Subtle variations in the specificity and the behavior of the enzymes from the two sources serve to focus upon these contrasts, with the hope that if the over-all reaction is the general theme, then the variations will bring about a better understanding.

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