Comparative Studies on ATP Production from Adenosine by a Methanol Yeast

Tetsu YONEHARA* and Yoshiki TANI

Research Center for Cell and Tissue Culture, Kyoto University,
Kyoto 606, Japan

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An ATP production system involving the oxidation of methanol, oxidative phosphorylation of ADP and phosphorylation of adenosine with sorbitol-treated cells of *Candida boidinii* (*Kloeckera* sp.) No. 2201 was studied with regard to the metabolism of adenosine and the role of sorbitol.

Analysis by high performance liquid chromatography showed the accumulation of IMP in the reaction mixture, possibly due to catalysis by AMP deaminase. The efficiency of energy conversion from methanol to nucleotides was improved 30% by preventing the evaporation of methanol. Among osmoregulatory compounds that plasmolyzed the yeast cells, only sorbitol caused high ATP production activity on addition to the reaction mixture. The sorbitol effect was attributed to the supplying of NADH by the oxidation in parallel with the methanol oxidation.

The ability of ATP production from adenine and adenosine was found in many methanol yeasts belonging to the genera, *Hansenula*, *Pichia*, *Candida* and *Torulopsis*. *C. boidinii* had especially high ability to accumulate ATP from adenosine.

In the course of investigations concerned with the industrial application of methylotrophs, we developed an ATP production system, from AMP,^{1~3)} adenosine⁴⁾ or adenine,⁴⁾ using cells of a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. The system involves the oxidation of methanol to reduce NAD⁺, the oxidative phosphorylation of ADP and the salvage synthesis of ADP. Efficient ATP accumulation was achieved from AMP³⁾ and adenosine⁴⁾ with sorbitol-treated cells with conversion rates of 70% and 60%, respectively.

In this study, we investigated the ATP production system of *C. boidinii* No. 2201 with regard to the metabolism of adenosine as a substrate and the role of sorbitol, and detected the ATP-producing activity in other methanol yeasts.

MATERIALS AND METHODS

Strains and chemicals. *C. boidinii* No. 2201, *C. boidinii* S-

1, *C. boidinii* S-2, *Candida* sp. 25-A, *Candida* sp. 1-A and *Saccharomyces sake* used were strains stored in our laboratory. Other yeasts were kindly supplied by the following: *Saccharomyces* sp. H-1 and *Candida* sp. N-16 from the Fermentation Research Institute, Ibaraki, *Hansenula capsulata*, *H. nonfermentans*, *H. glucozyma*, *Pichia trehalophila*, *P. halophila*, *P. pastoris*, *Candida cariosilignicola*, *C. succiphila*, *C. lipolytica*, *Torulopsis pinus* and *T. glabrata* from the Institute for Fermentation, Osaka, *Hansenula polymorpha* from Dr. Cooney, Massachusetts Institute of Technology, U.S.A., *Candida boidinii* from Dr. Sahm, (formerly) Gesellschaft für Molekularbiologische Forschung, West Germany, *Hansenula ofunaensis* and *Torulopsis nagoyaensis* from Mitsui Toatsu Chemicals Inc., Yokohama, *Pichia aganobii* from Mitsubishi Gas Chemical Co. Inc., Niigata, and *Candida methanolica* and *Torulopsis methanolovescens* from Sanraku Ocean Co., Ltd., Fujisawa. Cultivations were performed as described previously.⁴⁾ All chemicals were obtained from usual commercial sources and used without further purification.

Preparation of sorbitol-treated cells. Washed and sorbitol-treated cells were prepared as described previously.²⁾

Assay system for ATP production. The standard reaction mixture contained 25 μ mol K₂HPO₄, 50 μ mol Na₄P₂O₇.

* On leave from Toray Industries, Inc., Nagoya 455-91, Japan.

10H₂O, 20 μ mol adenosine, 5 μ mol NAD⁺, 5 μ mol reduced glutathione (GSH), 500 μ mol methanol, 40 μ mol MgSO₄·7H₂O, 300 μ mol sorbitol and 10 mg, as dry weight, of sorbitol-treated cells of *C. boidinii* No. 2201 in a total volume of 0.5 ml. The reaction was carried out and terminated as described previously.⁴⁾

The air-sealed reaction was initiated by addition of 0.2 ml cell suspension containing 300 μ mol sorbitol, followed by shaking obliquely in a 21-mm ϕ test tube containing 70 ml of air and sealed with a double rubber stopper. Other conditions were the same as the standard conditions.

When the activity was compared between different yeasts, the reaction mixtures contained 150 μ mol potassium phosphate buffer (pH 7.3), 20 μ mol adenosine or 5 μ mol adenine, 0.5 μ mol NAD⁺, 1.5 μ mol ATP, 40 μ mol MgSO₄·7H₂O, 500 μ mol methanol, 300 μ mol sorbitol and 10 mg, as dry weight, of sorbitol-treated cells of each yeast in a total volume of 0.5 ml. Other conditions were the same as the standard conditions.

Analyses. All analyses were carried out with the supernatants of the reaction mixtures, prepared as described previously.⁴⁾ ATP, ADP and AMP were analyzed by the enzymatic methods described previously⁴⁾ or by high performance liquid chromatography (HPLC). Other nucleotidic compounds were analyzed by HPLC.

HPLC was carried out with a Hitachi liquid chromatograph system 655 under the following conditions: column, M&S PACK C18 (4.6 mm i.d. $\times$ 150 mm); mobile phase, 0.2 M potassium phosphate buffer, pH 6.0 (solvent A), and methanol (solvent B); gradient, 2% of solvent B (0~6 min) and 10% of solvent B (6~20 min); flow rate, 1.0 ml/min; injection volume, 5 μ l; detection, UV (260 nm).

Methanol consumption was determined by gas chromatography as described previously.⁴⁾

Sorbitol was analyzed by the enzymatic method with sorbitol dehydrogenase.⁵⁾

RESULTS AND DISCUSSION

HPLC analysis of the reaction mixture for ATP production

The decrease in the total amount of adenine nucleotides in the reaction mixture with cells of *C. boidinii* No. 2201 was determined by differential enzymatic analyses of ATP, ADP and AMP. To determine the overall metabolism of the substrate, adenosine, is essential for the characterization of the ATP-producing system. Therefore, we developed an HPLC method for determining all nucleotidic compounds found in the reaction mixture. The HPLC method described under MATERIALS

FIG. 1. High Performance Liquid Chromatography of the Adenosine Phosphorylation Reaction Mixture.

The reaction mixture was analyzed as described under MATERIALS AND METHODS. Elution order: peak 1, IMP; peak 2, ATP; peak 3, ADP; peak 4, hypoxanthine; peak 5, xanthine; peak 6, AMP; peak 7, adenine; peak 8, NAD⁺; peak 9, inosine; peak 10, NADH; peak 11, adenosine.

AND METHODS made the simultaneous analysis of nucleotidic compounds possible. Figure 1 shows a chromatogram of a reaction mixture with *C. boidinii* No. 2201 cells, in which ATP, ADP, AMP, adenosine, adenine, IMP, inosine, hypoxanthine, xanthine, NAD⁺ and NADH were detected. The amount of each adenine nucleotide determined on HPLC analysis was confirmed to coincide with the enzymatic value.

Metabolism of adenosine in the ATP-producing reaction

Figure 2 shows a typical time course of ATP production by *C. boidinii* No. 2201 cells under the standard reaction conditions. ATP increased rather gradually as compared with adenosine consumption until 10-hr incubation. AMP and ADP were not found in the reaction mixture. This might show that the activity of adenosine kinase is rate-limiting for the production of ATP at this stage. At 10~14-hr incubation, AMP and ADP appeared, and total amount of adenine nucleotides coincided with that of adenosine consumed. After 14-hr incubation, IMP appeared and increased

FIG. 2. Time Course of Adenosine Metabolism under the Standard Reaction Conditions.

The reaction was carried out as described in the text as the established method: —●—, ATP; —■—, ADP; —▲—, AMP; —●—, adenosine; —△—, IMP; —□—, inosine.

rapidly. ATP accumulation continued until 22 hr. Further incubation increased the amounts of IMP and inosine.

No inosine appeared until 40 mM adenosine had disappeared. The total amount of ATP, ADP, AMP, adenosine, IMP and inosine always remained constant at 40 mM until 40-hr incubation.

From the results, it was concluded that the degradation of adenine nucleotides might be due to the activity of AMP deaminase and not to that of adenosine deaminase or adenine deaminase.

Efficiency of energy conversion from methanol

In previous studies,^{3,4)} we investigated and discussed the energy efficiency of the ATP production system with *C. boidinii* No. 2201 cells, which use methanol as an energy source, and the theoretical value, $Ke(t)$, for the conversion of adenosine to ATP was estimated to be 14.3%.⁴⁾ The ratio of the practical energy efficiency, $Ke(p)$, against the theoretical efficiency was only 2.2%, even though it included the energy of phosphate bonds of AMP and ADP besides ATP.⁴⁾ Here, we estimated the amounts of not only adenine nucleotides

but also that of IMP as energy stored compounds in the reaction mixture. Furthermore, the evaporation of methanol from the reaction mixture was prevented by the air-sealed system.

Table I shows the amounts of consumption of methanol and phosphorylation of adenosine in the reaction mixture. The practical energy efficiency against the methanol consumption expressed as follows,

$$Ke(p) = (2,181/51,302) \times 100 = 4.25\%$$

The ratio of the practical efficiency against the theoretical efficiency expressed as follows,

$$Ke = (Ke(p)/Ke(t)) \times 100 \\ = (4.25/14.3) \times 100 = 30\%$$

These values seem to be more reasonable compared with the previous ones.⁴⁾

Role of sorbitol in the ATP-producing system

The function of sorbitol in the ATP production system, from AMP, might be to make and maintain the plasma-membrane of *C. boidinii* No. 2201 cells permeable to nucleotidic compounds.²⁾ Some osmoregulatory compounds other than sorbitol could also be used during the preparation of plasmolyzed cells. These compounds, however, could not replace sorbitol in the ATP-producing reaction. This suggests that sorbitol has another function in the reaction mixture.

1) *Treatment of cells with sorbitol.* Table II shows the effect of each step in the preparation of sorbitol-treated cells on the ATP-producing activity. The treatment at 37°C before and after sorbitol addition could be replaced by a cold shock at -80°C. The addition of sorbitol was essential for the ATP-producing activity. Thus, the heat treatment might enhance the function of sorbitol in a kind of plasmolysis of the yeast cells.

2) *Replacement of sorbitol by other osmoregulatory compounds.* We tested other osmoregulatory compounds in the same way as sorbitol as to both cell treatment and ATP production. As shown in Table III, these compounds other than sorbitol could not make

TABLE I. ENERGY BALANCE OF ADENOSINE PHOSPHORYLATION

Energy source & product	Concn. (mM)	Reaction	ΔG (KJ/mol)	ΔG (J/liter)
Methanol consumed	73	Methanol $\rightarrow$ CO ₂	-702.8	-51,302
ATP produced	21.5	Adenosine $\rightarrow$ ATP	75.3	1,620
ADP produced	8.3	Adenosine $\rightarrow$ ADP	44.8	373
AMP produced	2.3	Adenosine $\rightarrow$ AMP	14.2	33
IMP produced	10.9	Adenosine $\rightarrow$ AMP $\rightarrow$ IMP	14.2 ^a	155
Energy-stored phosphates (bound in nucleotides)	94.3			2,181

^a On the assumption that IMP has the same free energy as AMP.

TABLE II. EFFECTS OF THE STEPS OF SORBITOL TREATMENT OF *C. boidinii* No. 2201 CELL ON ADENOSINE PHOSPHORYLATION ACTIVITY

The complete sorbitol treatment process²⁾ comprises 1) pre-heat treatment at 37°C for 45 min, 2) addition of sorbitol to a final concentration of 1.5M, and 3) heat treatment at 37°C for 10 min. The treated cells could be stored at -80°C. The activity was determined under the standard reaction conditions. The reaction time was 24 hr.

Step of treatment ^a				Relative activity ^b (%)
Pre-heat treatment	Sorbitol addition	Heat treatment	Stored at -80°C	
+	+	+	-	100
+	+	+	+	86.0
-	+	-	-	1.0
-	+	-	+	49.9
+	-	+	+	0
-	-	-	+	0

^a - and + denote that the step was omitted and carried out, respectively.

^b The activity was expressed as the amount of total nucleotides including ATP, ADP, AMP and IMP.

yeast cells show a high phosphorylating activity in the reaction. The addition of high concentrations of sorbitol or other reagents caused the death of yeast cells. The results showed that the viability of the yeast after both cell treatment and ATP production was not necessary for the activity. On the other hand, fructose and glucose could make cells show a weak phosphorylating activity in the same way as sorbitol. Especially, 0.4 M fructose

showed 8.5% relative activity compared with 0.6M sorbitol in the reaction. This suggests that sorbitol plays another role in the reaction mixture.

3) *Adenosine phosphorylation in a sorbitol-containing reaction mixture.* The probable other role of sorbitol in the reaction mixture was studied with the omissions and additions shown in Table IV. NAD⁺ and air were essential not only for the phosphorylation of adenosine but also the consumption of sorbitol. Fructose and glucose, especially fructose, were converted to sorbitol during the reaction without sorbitol. These results indicate the presence of a NAD⁺-dependent sorbitol dehydrogenase in the yeast cells. Omission of methanol increased the consumption of sorbitol. Figure 3 shows that the activity of adenosine phosphorylation decreased with increasing methanol concentration over the optimum concentration, but the consumption of sorbitol decreased with increasing methanol concentration.

From the results mentioned above, it might be concluded that the concentrations of NADH and NAD⁺ are balanced by coupling of NAD⁺-dependent oxidations of sorbitol to fructose and of methanol to CO₂, and NADH-dependent reduction of fructose to sorbitol. Thus, the additional role of sorbitol in the reaction mixture might be to control the amount of NADH in addition to osmoregulation (Fig. 4).

TABLE III. EFFECTS OF TREATMENT WITH OSMOREGULATORY COMPOUND ON CELL VIABILITY AND ADENOSINE PHOSPHORYLATION

Cells of *C. boidinii* No. 2201 were treated as described in the text, replacing 1.5 M sorbitol by each compound at the concentration indicated. The activity was determined under the standard reaction conditions except for the addition of each compound instead of sorbitol. The incubation time was 24 hr.

Compound	Concentration in		Cell viability (%)		Relative activity ^a (%)
	Cell treatment	Reaction mixture	Before reaction	After reaction	
None			100	3.1	0
Sorbitol	1.5 M	0.6 M	0.5	3.1	100
KCl	1.5 M	0.6 M	0	0	0
NaCl	1.5 M	0.6 M	0	0	0
Polyethylene glycol #4000	10%	4%			0
	15%	6%	20.7	4.7	0
	30%	12%			0
Ethylene glycol	1.0 M	0.4 M			0
	1.5 M	0.6 M	6.3	3.1	0
	3.0 M	1.2 M			0
Glycerol	1.5 M	0.6 M	0.9	1.1	0
Glucose	1.0 M	0.4 M			4.2
	1.5 M	0.6 M	0.2	0.8	0
Fructose	1.0 M	0.4 M			8.5
	1.5 M	0.6 M	0	1.2	2.5
Saccharose	0.75 M	0.3 M	1.8	3.6	0
Starch	15%	6%	5.3	1.9	0

^a Activity is expressed as in Table II.

TABLE IV. METABOLISM OF SORBITOL IN THE REACTION MIXTURE

Cells of *C. boidinii* No. 2201 were treated as described in the text. The standard reaction conditions were used except for the concentration of each compound. The incubation time was 24 hr.

Compound		Sorbitol (mm)		Relative activity ^a (%)
Omitted from reaction mixture	Added to reaction mixture	Consumed	Produced	
None	None	100.6	0	100
NAD ⁺	None	4.1	0	2.1
Air	N ₂	Trace	0	0
Methanol	None	303.0	0	35.5
Sorbitol	None	0	0	0
Sorbitol	0.6 M fructose	0	82.4	2.5
Sorbitol	0.4 M fructose	0	44.2	8.5
Sorbitol	0.6 M glucose	0	10.6	0

^a Activity is expressed as in Table II.

ATP productivity in methanol yeasts

So far, the ATP production system has been investigated only with *C. boidinii* No. 2201. The results, however, revealed that the activity

should be common among methanol yeasts. Twenty-four strains of methanol yeasts were tested as to their ATP productivity. It was found that NAD⁺ was gradually degraded to

TABLE V. ATP PRODUCTIVITY IN METHANOL YEASTS

Cells of each yeast were treated as described in Table II. The reaction and ATP analysis were carried out as described under MATERIALS AND METHODS. The reaction time was 22 hr.

Species	Strain	ATP produced from	
		Adenosine	Adenine
		(mM)	
(Ascomycetous yeasts)			
<i>Saccharomyces</i> sp. H-1		2.9	0.5
<i>Hansenula capsulata</i>	IFO 0974	16.0	3.8
<i>nonfermentans</i>	IFO 1473	3.9	1.7
<i>polymorpha</i>		1.0	tr**
<i>ofunaensis</i>		1.5	tr
<i>glucozyma</i>	IFO 1472	tr	tr
<i>Pichia trehalophila</i>	IFO 1683	1.6	1.2
<i>aganobii</i> Y-1023		11.8	1.7
<i>halophila</i>	IFO 0947	tr	tr
<i>pastoris</i>	IFO 0948	0.4	tr
<i>Saccharomyces sake</i> *	AKU 4111	tr	tr
(Asporogenous yeasts)			
<i>Candida boidinii</i>			
(<i>Kloeckera</i> sp.) No.2201	AKU 4705	27.2	7.7
<i>boidinii</i> S-1	AKU 4614	17.5	4.8
<i>boidinii</i> S-2	AKU 4615	11.9	3.2
<i>boidinii</i>		4.6	2.0
sp. N-16		1.8	tr
sp. 25-A	AKU 4621	21.8	2.3
sp. 1-A	AKU 4622	22.3	1.6
<i>methanolica</i>	ATCC 26175	12.3	2.3
<i>cariosilignicola</i>	IFO 1910	4.9	1.3
<i>succiphila</i>	IFO 1911	5.8	1.6
<i>Torulopsis pinus</i>	IFO 0741	tr	tr
<i>pinus</i>	IFO 1327	2.9	1.2
<i>methanolovescens</i>	ATCC 26176	10.0	2.3
<i>nagoyaensis</i>		3.1	1.6
<i>glabrata</i>	IFO 0005	2.1	tr
<i>Candida lipolytica</i> *	IFO 0717	1.7	tr

* These yeasts were used as references.

** tr (0.1 mM).

supply ATP until 10-hr incubation and then recovered with the accumulation of more than 3 mM ATP under the standard reaction conditions with *C. boidinii* No. 2201 cells (unpublished data). This might cause errors on comparison of ATP productivity. Therefore, we compared the activity of these yeasts using the reaction conditions given under MATERIALS AND METHODS, under which the effect of the NAD⁺ concentration on the activity was minimized.

As shown in Table V, significant activity of ATP production from 40 mM adenosine or 10 mM adenine was found in several strains of methanol yeasts, e.g. *H. capsulata*, *P. aganobii*, *C. boidinii* S-1, *C. boidinii* S-2, *C. methanolica*, *T. methanolovescens* and so on. Most strains belonging to the genus, *Candida*, showed high ability to produce ATP from adenosine. Among them, *C. boidinii* No. 2201 showed the highest productivity of ATP from not only adenosine but also adenine un-

FIG. 3. Effect of Methanol on Energy Source Utilization.

The reaction was carried out with the air-sealed reaction system except for the concentration of methanol and the feeding of 150 mM potassium phosphate buffer (pH 7.3) at 15-hr incubation. The incubation time was 26 hr.

der the conditions employed.

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FIG. 4. Control System of the NADH Concentration by Sorbitol Dehydrogenase.

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