Phosphorylation of Mononucleotides and Formation of Cytidine 5′-Diphosphate-Choline and Sugar Nucleotides by Respiration-Deficient Mutants of Yeasts

AKIRA KIMURA,* KAZUHIKO HIROSE, YASUHIRO KARIYA, AND SUSUMU NAGAI
Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606,* and Faculty of Science, National Women’s University, Nara 630, Japan

Received for publication 29 September 1975

Respiration-deficient mutants (Rho−, petite) of Saccharomyces carlsbergensis were obtained by treatment with trypanflavin (euflavine). Dried cells of these mutants phosphorylated mononucleotides to their triphosphates and further formed not only cytidine 5′-diphosphate-choline, but also sugar nucleotides, such as uridine 5′-diphosphate-glucose, guanosine 5′-diphosphate-mannose, etc. The activities were the same or slightly greater than those of the wild strain. These results showed that energy (adenosine 5′-triphosphate) necessary for phosphorylation of mononucleotides was sufficiently supplied by the glycolysis system.

Cytidine 5′-diphosphate-choline (CDP-choline) is not only an important intermediate in the biosynthesis of phospholipids, but also an effective drug for various kinds of brain injuries. Therefore, an inexpensive means of producing CDP-choline would be valuable. We succeeded in producing it by using dried cells of many yeasts (12) and later by using intact cells of a yeast, Saccharomyces carlsbergensis, treated with Triton X-100 (4). CDP-choline was formed from phosphorylcholine and cytidine 5′-monophosphate (CMP), which had been a waste product of the nucleotide industry in Japan.

The mechanisms of CDP-choline formation have been intensively investigated (5, 6). The process resulted in a variety of phosphorylations. Not only CMP, but also other mononucleotides, were phosphorylated in the same way by the energy (adenosine 5′-triphosphate [ATP]) generated by yeasts, when an adequate amount of glucose, phosphate, etc. was added to the reaction mixture. Generally, ATP is generated by glycolysis and by the respiratory system. The former generates 2 mol of ATP from 1 mol of glucose, whereas the latter generates 36 mol of ATP. It was of interest to decide whether ATP produced through the glycolysis system is used for the phosphorylation of mononucleotides (uridine 5′-mononucleotide [UMP], cytidine 5′-mononucleotide [CMP], guanosine 5′-mononucleotide [GMP], and adenosine 5′-mononucleotide [AMP]) or whether ATP generated by the respiratory system is indispensable. To solve this problem, we used respiration-deficient (RD) mutants (7, 9) of Saccharomyces carlsbergensis, which contained only cytochrome c (1), lacking cytochromes a and b (2), and did not take up any oxygen. The three RD mutants (defined as pet-1, pet-2, and pet-3) could not grow on glycerol medium and formed white colonies on tellurite medium (8). They were cultured for 4 days in medium containing 5% glucose, 0.3% peptone, 0.1% yeast extract, 0.2% KH2PO4, 0.3% (NH4)2HPO4, and 0.1% MgSO4·7H2O. To identify the RD mutants, 0.5 mg of 2,3,5-triphenyl-tetrazolium chloride per ml (Nakarai Chemicals Ltd., Kyoto, Japan), together with 5 mg of glucose per ml and 1% agar, was overlaid on colonies in petri dishes (9). After harvest, cells were dried with an electric fan as described in previous papers (5, 6, 11) and incubated in 2 ml of the reaction mixture containing 800 mM glucose, 20 mM mononucleotide, 200 mM potassium phosphate buffer (pH 7.0), and 12 mM MgSO4·7H2O, and 50 mM phosphorlycholine-calcium salt. A 200-μg portion of dried cells was used in 2 ml of the above-mentioned reaction mixture. After the reaction time indicated, the reaction was stopped by placing the reaction tube in boiling water. The samples were centrifuged at 3,500 × g for 5 min, and the supernatant fluids were subjected to paper chromatography. The paper chromatograms were developed with a mixed solvent system containing 95% ethanol and 1 M ammonium acetate (2:1, vol/vol, pH 7.5) (6, 12). All nucleotides and their derivatives were determined by measuring their optical densities at 260 and/or 280 nm after extraction with 0.01 N HCl from paper chromatograms.
Using the RD mutants obtained, we tested phosphorylation of mononucleotides. CMP was phosphorylated to cytidine 5'-triphosphate (CTP) and then converted to CDP-choline (Fig. 1). The RD mutants, as well as the wild strain, transformed about 60% of CMP to CDP-choline. From this result we could conclude that the energy (ATP) produced from the glycolytic system is quite enough to phosphorylate CMP and to produce CDP-choline. We have further shown that the RD mutants could phosphorylate other nucleotides such as UMP (Fig. 2), GMP (Fig. 3), and AMP (Table 1), and that they could form sugar nucleotides. Mutant pet-2 showed slightly different behavior from other mutants in that it decomposed UMP and GMP more vigorously to uracil and guanine, respectively. Since these reactions were carried out under the same conditions (800 mM glucose, 200 mM potassium phosphate, 12 mM MgSO₄, and 20 mM nucleotides), the yields of phosphorylation of each nucleotide were not always maximal. AMP was decomposed under these conditions, but it was also phosphorylated to ATP (Table 1) when the concentration of potassium phosphate was increased from 200 to 800 mM.

Recently a ubiquinone-less mutant of Saccharomyces cerevisiae was isolated by De Kok and Slater (3). Using this strain, we examined phosphorylation of CMP by dried cells and found that this mutant also could phosphorylate CMP and that it produced almost the same amount of CDP-choline as a wild strain. As will be discussed later, the development of the respiratory chain did not seem to be required for these phosphorylations. Nevertheless, ATPs produced by glycolysis and by the respiratory chain might be distinguished by the cells. This assumption may be supported by the fact that dried cells of Hansenula jadinii, one of the best producers of CDP-choline (11), completely lost their activity to phosphorylate mononucleotides when they were cultivated in a jar fermentor under aerobic conditions, although the cells grew vigorously. Since growing cells must obviously be able to convert mononucleotides to the triphosphates for nucleic acid synthesis, the
adenosine, ADP, \( \text{pet-3} \)

amount of

conditions \( \text{pet-3} \)(D), \( \text{mM} \).

5'-diphosphate

strain (A) of (guanosine, guanine, \( \text{pet-1} \), \( \text{pet-2} \), \( \text{pet-3} \) (D), is shown together with that of the wild strain (A) of \( \text{S. carlsbergensis} \). Symbols: \( \bigcirc \), GMP; \( \bigcirc \), GDP+GTP; \( \bigcirc \), GDP-mannose; \( \big\Delta \), side products (guanosine, guanine, etc.).

![Graph showing phosphorylation of GMP and formation of guanosine 5'-diphosphate (GDP)-mannose by RD mutants of \( \text{S. carlsbergensis} \).](image)

**FIG. 3. Phosphorylation of GMP and formation of guanosine 5'-diphosphate (GDP)-mannose by RD mutants of \( \text{S. carlsbergensis} \).** Formation of GDP-mannose by three RD mutants, pet-1 (B), pet-2 (C), and pet-3 (D), is shown together with that of the wild strain (A) of \( \text{S. carlsbergensis} \). Symbols: \( \bigcirc \), GMP; \( \bigcirc \), GDP+GTP; \( \bigcirc \), GDP-mannose; \( \big\Delta \), side products (guanosine, guanine, etc.).

**TABLE 1. Phosphorylation of AMP by RD mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Reaction time (h)</th>
<th>AMP (micromoles)</th>
<th>ADP + ATP (micromoles)</th>
<th>Hypoxanthine and inosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pet-1</td>
<td>0</td>
<td>19.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>11.0</td>
<td>9.0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.0</td>
<td>12.4</td>
<td>3.4</td>
</tr>
<tr>
<td>pet-2</td>
<td>0</td>
<td>20.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.3</td>
<td>15.8</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.5</td>
<td>12.3</td>
<td>1.3</td>
</tr>
<tr>
<td>pet-3</td>
<td>0</td>
<td>20.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.3</td>
<td>13.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.0</td>
<td>13.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

![Graph showing phosphorylation of AMP by RD mutants](image)

*The reactions were carried out under the usual conditions except for phosphate concentration (800 mM). RD mutants of \( \text{S. carlsbergensis} \) were used. ADP, adenosine 5'-diphosphate. Values show the amount of compound in micromoles per milliliter.

failure to demonstrate in vitro phosphorylation might be explained either by failure to penetrate the cells or by compartmentalization (10).

We want to express our sincere thanks to T. Tochikura for his kind support and encouragement. Much helpful information regarding phosphorylation of AMP from T. Yano of our laboratory is acknowledged with gratitude. We also want to express our sincere thanks to J. De Kok for his kind supply of valuable strains.

**LITERATURE CITED**


