ENZYMIC RIBONUCLEOSIDE REDUCTION AT THE NON-PHOSPHORYLATED LEVEL IN ACETABULARIA

EGON J. DE GROOT AND HANS-GEORG SCHWEIGER
Max-Planck-Institut für Zellbiologie, 6802 Ladenburg bei Heidelberg, West Germany

SUMMARY
The occurrence of an enzyme that catalyses the conversion of cytidine into deoxycytidine was demonstrated in homogenates of Acetabularia. Cytidine was identified as the substrate by comparing cytidine, cytidine 5'-monophosphate, cytidine 5'-diphosphate and cytidine 5'-triphosphate as potential substrates. Experiments with ATP analogues whose inhibitory effect on kinase reactions is well established, supplied evidence that the nucleoside is reduced without a phosphorylation step before the reduction. Further evidence in this line came from incubations with cytidine in the presence of phosphatase and from trap-type experiments in which the effects of excess non-labelled cytidine 5'-phosphate and deoxycytidine, respectively, on the formation of deoxycytidine phosphates from cytidine were studied.

INTRODUCTION
Enzymes that convert the ribose moiety of nucleotides into deoxyribose are very important because all deoxyribonucleotides, particularly those of DNA, have to pass through this enzymic reaction (Larsson & Reichard, 1967; Reichard, 1967; Blakley & Vitols, 1968; Follmann, 1974; Hogenkamp & Sando, 1974; Thelander & Reichard, 1979). It is generally accepted that reduction occurs at the nucleoside diphosphate level (Larsson & Reichard, 1967; Thelander & Reichard, 1979; Reichard, 1968). Nucleoside triphosphates have been identified as substrates for reduction in only a few prokaryotic organisms (Abrams, 1965; Gleason & Hogenkamp, 1972; Hamilton, 1974; Munavalli, Parker & Hamilton, 1975; Blakley, 1978).

In the unicellular and uninucleate marine green alga Acetabularia (for references, see Schweiger, 1969; Schweiger & Berger, 1979) the number of nuclei is substantially increased to a total of \(10^6\) or even more during the generative phase of its life cycle (Schweiger, Berger, Kloppsteck, Apel & Schweiger, 1974). Closely related to this high rate of nuclear division is the concomitant regulation of a number of enzymes that are involved in deoxyribonucleotide metabolism. Among these enzymes are a dCMP-deaminase (Bannwarth, Ikehara & Schweiger, 1982), a thymidine kinase (Bannwarth & Schweiger, 1975; Bannwarth, Ikehara & Schweiger, 1977a,b) and a thymidylate kinase (de Groot & Schweiger, 1983a,b). It

Key words: Acetabularia, cytidine reductase.
E. J. de Groot and H.-G. Schweiger

has been shown that these enzymes are regulated by de novo synthesis rather than by the activation of a pre-existing enzyme protein. On the basis of inhibitor studies it was concluded that in Acetabularia the first two enzymes are products of an organellar gene expression system while the third enzyme is translated on 80 S ribosomes (Bannwarth et al. 1977a,b, 1982; Bannwarth & Schweiger, 1975; de Groot & Schweiger, 1983b).

Because of the crucial role of the reductase a study was begun to investigate the behaviour of the enzyme during the early generative phase of Acetabularia. This paper demonstrates an enzymic reduction reaction in Acetabularia that starts with a ribonucleoside as substrate.

MATERIALS AND METHODS

Cells

Cells of Acetabularia mediterranea were grown either in Erd-Schreiber medium (Schweiger, 1969; Hämerling, 1963) or in a fully defined artificial medium (Müller, 1962; Berger & Schweiger, 1980).

Cell homogenate

A hundred cells were selected, washed at least twice with Müller medium, and blotted on filter paper. The cells were frozen and thoroughly ground with a pestle and mortar in liquid nitrogen. The frozen powder was thawed by adding 3 ml of 0.1 M-Tris HCl buffer (pH 7.5) containing 10 mM-ATP and 20 mM-MgCl2 (incubation buffer).

Enzyme assay

The homogenate was used as the enzyme preparation. Protein content was determined according to the method of Lowry, Rosebrough, Farr & Randall (1951). The reaction was started by adding 30 μl of the substrate ([5-3H]cytidine, sp. act. 268 Ci mmol−1, New England Nuclear) to 3 ml of the enzyme preparation. The mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 3 ml of 1 M-HClO4. Cytidine diphosphates and triphosphates were hydrolysed to the corresponding monophosphates by heating the mixture for 30 min at 100°C. The mixture was then neutralized with 4 M-KOH and stored at −20°C.

Separation of nucleotides was performed by means of cation exchange chromatography. Following thawing of the mixture, precipitated KClO4 and protein were removed by centrifugation for 15 min at 4500 g. The supernatant was used for analysis of the reaction products. After addition of 250 μl of a solution of CMP, dCMP, dC and C (10 mM each) the reaction products were separated on a 0.9 cmX50 cm column of AG 50W-X8 (200-400 mesh, hydrogen form) (Reichard, 1958). CMP and dCMP were eluted and separated with 625 ml 0.2 M-CH3COOH, whereas C and dC were eluted and separated with 350 ml 1 M HCl. The flow rate was 0.4 ml min−1. The absorbance of each 5 ml volume was measured at 280 nm. Radioactivity was estimated in 1-ml samples. Liquid scintillation counting was performed after addition of 10 ml Packard emulsifier 299 cocktail. Identification of the reaction products dCMP and dC was performed by thin-layer chromatography on cellulose plates MN300 (Machery & Nagel) using 0.02 M-HCl in the first direction and a 1:1 (v/v) mixture of 95% ethanol and 1 M-CH3COONa saturated with Na2B4O7·10 H2O in the second direction (Rossi & de Petrocellis, 1975). Compounds dCMP, CMP, dC and C were located with an ultraviolet lamp. The thin-layer plate was cut into 0.5-cm pieces and radioactivity was measured in a scintillation counter by adding 10 ml Packard emulsifier 299 cocktail. In both methods authentic non-radioactive reference material coeluted and comigrated, respectively, with the labelled products.

In some experiments acid hydrolysis was replaced by incubation with alkaline phosphatase. In
Non-phosphorylated ribonucleoside reduction

this case the reaction mixture was heated for 10 min at 100°C after incubation with labelled cytidine. After addition of 70 units of calf intestine alkaline phosphatase and incubation overnight at 25°C all nucleotides were converted to the corresponding nucleosides.

RESULTS

With cytidine diphosphate as the substrate, essentially no reductase activity could be detected after incubation with the homogenate. This could not be explained by poor separation techniques, since the cation exchange chromatography used to separate C, dC, CMP and dCMP turned out to be appropriately efficient and since cytidine was accepted as the substrate under the same conditions.

In order to estimate not only the products listed but also their diphosphate and triphosphate derivatives, the samples were subjected to acid hydrolysis or to phosphatase treatment before cation exchange chromatography, resulting in the formation of monophosphates and nucleosides, respectively. After acid hydrolysis separation was performed by cation exchange chromatography, which allowed a distinction to be made between the reduced and non-reduced compounds. After phosphatase treatment C and dC were separated by means of anion exchange of the borate complexes (Cory, Russel & Mansell, 1973; Steeper & Steuart, 1970).

The first indication that neither the nucleoside disphosphate nor the nucleoside triphosphate is a substrate for enzymic reduction in homogenates from Acetabularia came from a comparison of the efficiency of cytidine, cytidine 5'-monophosphate, cytidine 5'-diphosphate and cytidine 5'-triphosphate as substrates, each of them labelled with tritium in the 5' position (Table 1). The compound that gave the highest yield of dCMP was the non-phosphorylated cytidine.

The significant yield of the monophosphate and the limited conversion of the diphosphate do not necessarily contradict the assumption that it is the nucleoside that is reduced, but rather might be explained by the presence of endogenous phosphatase activity in the homogenates (Spencer & Harris, 1964; Tripelett, Steens-Lievens & Baltus, 1965).

Table 1. Reductase activity of a homogenate from Acetabularia with various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conversion to deoxyribosides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[5-3H]cytidine</td>
<td>0.059</td>
</tr>
<tr>
<td>[5-3H]cytidine 5'-monophosphate</td>
<td>0.033</td>
</tr>
<tr>
<td>[5-3H]cytidine 5'-diphosphate</td>
<td>0.004</td>
</tr>
<tr>
<td>[5-3H]cytidine 5'-triphosphate</td>
<td>-</td>
</tr>
</tbody>
</table>

A total of 400 Acetabularia cells at the cyst stage were washed, blotted on filter paper, frozen in liquid nitrogen and ground with a pestle and mortar. The frozen powder was divided into four equal portions, by weight. Each portion was suspended in 5 ml incubation buffer. The homogenates were incubated at 37°C for 1 h in the presence of 30 μCi of either [5-3H]cytidine, [5-3H]cytidine 5'-monophosphate, [5-3H]cytidine 5'-diphosphate or [5-3H]cytidine 5'-triphosphate and subjected to acid hydrolysis. CMP and dCMP were separated by cation exchange chromatography.
Table 2. Theoretical possibilities of conversion of C into dC, dCMP, dCDP and dCTP

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>C → dC</td>
<td></td>
</tr>
<tr>
<td>CMP → dCMP</td>
<td></td>
</tr>
<tr>
<td>CDP → dCDP</td>
<td></td>
</tr>
<tr>
<td>CTP → dCTP</td>
<td></td>
</tr>
</tbody>
</table>

In early experiments in which the time-course of the labelling of dC, dCMP and dCDP was studied, labelled cytidine was used as the substrate. In these experiments the first product to reach maximum labelling was dC. In fact, the labelling of this compound was much faster than the labelling of dCMP and dCDP (results not shown).

Consideration of the possible pathways from cytidine to the different deoxy-nucleotides (Table 2) reveals that, with cytidine as substrate, the first step must be either a phosphorylation or a reduction. On the basis of this consideration a number of experiments were performed in order to decide which alternative is catalysed in the homogenate of Acetabularia and whether it is indeed the cytidine that is reduced without any phosphorylation step before reduction.

In the absence of ATP the reduction of cytidine is significantly diminished (results not shown). However, this result does not necessarily mean that reduction depends on phosphorylation of the substrate, since if ATP is replaced by analogues whose inhibitory effect on kinase reactions is well established, like AMP-PCP, AMP-PNP and AMP-S (Yount, Babcock, Ballantyne & Ojala, 1971a; Yount, Ojala & Babcock, 1971b), reductase activity remains almost constant, while the kinase reaction is substantially decreased (Table 3).

To answer the question of whether a phosphorylating step is a prerequisite for the reduction of cytidine, the reaction was studied in the absence and the presence of added phosphatase. If the phosphorylation step were essential for the reduction one would expect that, due to the added phosphatase, any CMP that was formed by

Table 3. Effect of ATP analogues on the cytidine reductase reaction

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration (mm)</th>
<th>Enzyme activity (disnts min⁻¹ cell⁻¹ 10⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>10</td>
<td>1·1</td>
</tr>
<tr>
<td>AMP-PCP</td>
<td>3</td>
<td>2·0</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>3</td>
<td>0·9</td>
</tr>
<tr>
<td>AMP-S</td>
<td>3</td>
<td>1·2</td>
</tr>
</tbody>
</table>

ATP was replaced by either AMP-PCP (adenyl-β, γ-methylene-diphosphonate); AMP-PNP (adenyl-imidodiphosphate); or AMP-S (adenosine-5'-o-thionophosphate) (for conditions see Table 1). Incubation was started by adding 30 μl of [5-³H]cytidine. The mixture was incubated at 37°C for 1 h (for conditions see Table 1).
Non-phosphorylated ribonucleoside reduction

Table 4. Effect of phosphatase on the cytidine reductase reaction

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme activity (disints min⁻¹ cell⁻¹ 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>− Phosphatase</td>
<td>2.3</td>
</tr>
<tr>
<td>+ Phosphatase</td>
<td>2.5</td>
</tr>
<tr>
<td>Heat-inactivated</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Homogenates (for conditions see Table 1) were supplemented and preincubated with 70 units of alkaline phosphatase for 30 min at 25°C. The reaction was started by adding 30 μl of [5-³H]cytidine. The mixtures were incubated at 37°C for 1 h. The enzyme reaction was stopped by heating the incubation mixtures for 10 min at 100°C. Substrate and reaction products were separated by anion exchange chromatography using Dowx 1-borate.

Table 5. Effect of an excess of unlabelled dC or CMP on the conversion of labelled C to dC derivatives

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration (mM)</th>
<th>Conversion to deoxyribosides (disints min⁻¹ cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>117</td>
</tr>
<tr>
<td>dC</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CMP</td>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>

Homogenates (for conditions see Table 1) were supplemented with either unlabelled dC (1 mM) or CMP (1 mM). The mixture was incubated at 37°C for 1 h in the presence of [5-³H]cytidine. The products were separated after acid hydrolysis.

Phosphorylation of C would be immediately dephosphorylated. However, the result clearly showed that the phosphatase did not influence the reduction of cytidine, again demonstrating that phosphorylation is not an essential prerequisite for reduction (Table 4).

Another approach allowing a distinction between the two alternative pathways starting with cytidine as substrate, and to answer the question of which comes first, reduction or phosphorylation, uses a trap-type experiment. In this experiment the effect of the addition of a high excess of unlabelled dC and CMP, respectively, on the conversion of labelled cytidine into dCMP, dCDP and dCTP was studied. One would expect that if dC is the first product in this metabolic pathway, the presence of an excess of unlabelled dC would suspend the labelling of dCMP, dCDP and dCTP. If, however, reduction was preceded by phosphorylation, unlabelled dC should not interrupt the labelling of the deoxyribonucleotides. Otherwise, in the presence of an excess of unlabelled CMP the flow of the label into the phosphorylated dC compounds would be suspended if a phosphorylation step were a prerequisite for the reduction, and the reduction would not be substantially affected if unphosphorylated C were reduced. The results show that in the presence of an excess of unlabelled dC the phosphorylated dC compounds did not contain any radioactivity (Table 5).
Table 6. Reductase activity in sediment and supernatant fraction

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme activity (disints min⁻¹ cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment 1</td>
<td>54</td>
</tr>
<tr>
<td>Sediment 2</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0</td>
</tr>
</tbody>
</table>

Homogenates from cells with cysts (for conditions see Table 1) were spun for 20 min at 20 000 g: sediment 1. The supernatant 1 was recentrifuged for 60 min at 100 000 g: sediment 2; supernatant 2. The sediment fractions were suspended in 7 ml incubation buffer. The reductase activity was estimated in the sediment and the supernatant.

Table 7. Washing and separating of the pellet after centrifugation of the homogenate

<table>
<thead>
<tr>
<th>Treatment of homogenate</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage at 0°C</td>
<td>38</td>
<td>33</td>
</tr>
<tr>
<td>Centrifugation, 3×,</td>
<td>33</td>
<td>20</td>
</tr>
<tr>
<td>resuspension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation, 3×,</td>
<td>42</td>
<td>34</td>
</tr>
<tr>
<td>separation of pellet</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Homogenates from cells with cysts (for conditions see Table 1) were divided into three equal portions: one portion was stored at 0°C, the other two were centrifuged (5 min at 4500 g). The pellet of one sample was resuspended in its own supernatant and recentrifuged twice, the other pellet was washed with buffer and also recentrifuged. The untreated homogenate and the two pellets were incubated simultaneously for 60 min at 37°C in the presence of [5-³H]cytidine.

On the other hand, in the presence of an excess of unlabelled CMP there was still a considerable flow of radioactivity into the deoxyribose compounds. The results lead to the conclusion that phosphorylation is not a prerequisite for reduction. The low rate of incorporation in the presence of unlabelled CMP might be explained by the presence of endogenous phosphatase activity in the homogenate, which would result in a dilution of the labelled substrate and a lower specific activity of the cytidine. This would then diminish the labelling of the reduced products.

Partition of the homogenate by centrifugation for 20 min at 20 000 g revealed that the enzyme is exclusively contained in the sediment (Table 6). This indicates that cofactors and other substrates are not involved in the enzymic reaction. In order to substantiate this finding further a sediment fraction was subjected to two additional washing steps. A comparison between non-centrifuged and centrifuged, resuspended but not separated homogenates showed unequivocally that no cofactors or other low molecular weight substrates are essential for the reduction (Table 7).

Finally, the dependence of the enzyme on pH (Fig. 1), temperature (Fig. 2) and magnesium was studied. The optima for pH and temperature are 7·4 and 37°C,
Fig. 1. Effect of pH on cytidine reductase activity. Homogenates were incubated at 37°C for 1 h in the presence of [5-3H]cytidine. Separation of the products was performed by cation exchange chromatography.

respectively. There is no dependence of the enzyme activity on magnesium in the range between 0 and 20 mM. Attempts to find a stimulation of reductase activity by adding reducing agents like NADH, NADPH and dithiothreitol were not successful, nor was the addition of reduced glutathione and glutathione reductase. The same is true for 5'-deoxyadenosylcobalamin and thioredoxin.

DISCUSSION

In contrast to what is generally accepted, namely that ribonucleoside compounds are reduced only at the level of ribonucleoside diphosphates and in some cases ribonucleoside triphosphates, the experiments described here clearly show that in *Acetabularia* cytidine is reduced without requiring a phosphorylation step. The
Fig. 2. Temperature dependence of cytidine reductase. Homogenates (for conditions see Table 1) were incubated for 1 h at the given temperature in the presence of [5-^3^H]cytidine. Separation of the products was performed by cation exchange chromatography.

Evidence is based on: the high yield with C as substrate, the lack of effect of ATP analogues and phosphatases on the reduction, and on trap-type experiments.

An alternative possibility would be as follows: the reduction of ribonucleosides is simulated by a particulate structure surrounded by a membrane that is permeable only to the unphosphorylated ribonucleoside. Inside the structure, reduction occurs after phosphorylation to the diphosphate. This possibility is however excluded since an enzyme preparation that was frozen and thawed three times, or was sonified, catalysed the reduction of C but not of CDP (results not shown).

The reductase of *Acetabularia* exhibits a major difference from the reductases of other organisms with respect to the requirement of cofactors. While the ribonucleoside triphosphate reducing enzyme exhibits a specific requirement for
Non-phosphorylated ribonucleoside reduction

5'-deoxyadenosylcobalamin (Hamilton, 1974; Blakley & Barker, 1964; Gleason & Frick, 1980) and the ribonucleoside diphosphate reductases depend on reducing substances, like thioredoxin (Larsson & Reichard, 1967) these substances do not affect ribonucleoside reductase activity in *Acetabularia*.

A discussion of the mechanism of reduction in *Acetabularia* should include two alternative pathways. More recently, it has been suggested that ribothymidine is phosphorylysed to thymine and ribose 1-phosphate (Lothrop & Uziel, 1982). In the presence of deoxyribose 1-phosphate the thymine could then be converted into thymidine by thymidine phosphorylase (Desgranges, Razaka, Rabaud & Bricaud, 1981). Such a pathway would require the production of deoxyribose 1-phosphate. Deoxyribose aldolase is an enzyme that might catalyse the synthesis of deoxyribose (Racker, 1951, 1952; Groth & Jiang, 1966; Turner, Abrams & Lieberman, 1966). However, whether this reaction can play a major role in the cell has been questioned (Reichard, 1967).

Two types of experiments that were performed (data not shown) in our laboratories have excluded the possibility that such a phosphorylase reaction occurs in the homogenate of *Acetabularia*. In the first experiment, the reaction was started from base-labelled cytidine, while unlabelled cytosine and deoxyribose 1-phosphate were added in excess. If a phosphorylase-catalysed step were the main source of deoxyribose compounds, deoxycytidine phosphates should not be labelled. The experiment clearly showed that in contrast to the prediction the labelling of the deoxycytidine phosphates was not impaired under these conditions. Moreover, if labelled cytosine was used as substrate, no labelled deoxycytidine phosphates could be detected after the enzymic reaction.

Another explanation for the fact that phosphorylation is not a prerequisite for the formation of deoxycytidine could be that the ribose moiety of the cytidine is exchanged with a deoxyribose moiety from, e.g., a deoxyribonucleoside by a transferase, according to the following scheme where X might, e.g., be another purine or pyrimidine base:

\[
\begin{array}{cccccc}
  C & X & C & X \\
  \mid & + & \mid & + & \mid \\
  R & dR & dR & R
\end{array}
\]

Such a reaction raises the question of the origin of the X-dR. Incubation in the presence of phosphatase rules out the possibility that X-dR is being formed by a nucleoside diphosphate reductase reaction. On the other hand, the presence of a significant pool of pre-existing X-dR in the enzyme preparation was excluded by the washing experiment.

An interesting question is what specific role a reductase would play in the cell if its substrate is a non-phosphorylated ribose and what its source might be. The precursor of the cytidine in any case would be the cytidine monophosphate and the cytidine substrate would be the product of a phosphatase reaction. One interesting possibility is that the enzyme is a salvage enzyme. As has been shown (de Groot &
Schweiger, 1985), the enzyme is strongly regulated during the early generative phase of the life cycle of *Acetabularia*, i.e. during that phase of the life cycle in which a tremendous number of nuclear divisions takes place. During this phase, the number of nuclei increases from 1 to $10^6$, or even more (Schweiger et al. 1974). It could well be that at the end of the vegetative phase the numbers of secondary nuclei, chloroplasts and mitochondria are disproportionate, which would mean that those organelles present in excess ought to be degraded. That would mean a significant amount of RNA is available for deoxyribonucleotide production via a salvage pathway.

The reduction of ribonucleosides in the cell is one of the crucial enzymic reactions preceding DNA replication (Thelander & Reichard, 1979; Eriksson & Martin, 1981). In our experiments it has been shown that in *Acetabularia* the activity even during the generative phase of the life cycle is pretty low, compared to other enzymes that are involved in deoxyribonucleotide metabolism. This might indicate that the enzyme plays a significant role in metabolic regulation. Unfortunately, all attempts to solubilize the enzyme without losing its activity have failed.

We thank Professors Horst Bannwarth and Eckhard Schlimme for fruitful discussions and Mr Douglas Fiebig for carefully reading the manuscript. The excellent technical assistance of Mrs Rita Menze and the careful preparation of the manuscript by Mrs Brigitte Nagel and Christiane Schardt is gratefully acknowledged.

REFERENCES


Non-phosphorylated ribonucleoside reduction


(Received 22 August 1984 – Accepted 24 August 1984)