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Recombinant *Lactobacillus leichmannii* ribonucleosidetriphosphate reductase as biocatalyst in the preparative synthesis of 2′-deoxyribonucleoside-5′-triphosphates

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Abstract

Recombinant *Lactobacillus leichmannii* ribonucleosidetriphosphate reductase has been purified and evaluated as a biocatalyst for the preparative synthesis of 2′-deoxyribonucleoside-5′-triphosphates. The addition of expensive 2′-deoxyribonucleoside-5′-triphosphates as allosteric effectors of ribonucleosidetriphosphate reductase was not necessary due to high concentrations of inorganic salts in the reaction mixture. Good conversion of the tested ribonucleoside-5′-triphosphate substrates ATP, CTP, GTP, ITP, and UTP was observed. From a variety of reducing agents 1,4-dithio-DL-threitol (DTT), 1,4-dithioerythritol (DTE), bis-(2-mercaptoethyl)-sulfone, and 1,3-propanedithiol showed to be the most effective reducing agents for re-reduction of the active center thiols of ribonucleosidetriphosphate reductase. The kinetic parameters of ribonucleosidetriphosphate reductase with respect to affinity of ribonucleoside-5′-triphosphate substrates, the cofactor 5′-deoxyadenosylcobalamin, and the reducing agents DTT or 1,3-propanedithiol under the employed reaction conditions were determined. Substrate inhibition was not observed. Preparative gram-scale 2′-reductions of ribonucleoside-5′-triphosphates proceeded to completion.

Keywords: Enzymatic synthesis; Ribonucleotide reductase; *Lactobacillus leichmannii*; 2′-deoxyribonucleoside-5′-triphosphate

1. Introduction

On the advent of antisense drugs in cancer treatment and other indication areas where suppression of gene translation by blocking of mRNA is a therapeutic strategy, a need for DNA building blocks on large scale may arise within a short period of time. Classic 2′-deoxyribonucleoside and 2′-deoxyribonucleoside-5′-monophosphate sources include mainly hydrolyzed herring and salmon milt DNA.

We investigated alternative approaches to the industrial production of 2′-deoxyribonucleosides. A purely chemical approach has shown to be too expensive, too complicated, or too unselective. An economic enzymatic synthesis strategy starting from cheap material like starch that is hydrolyzed and cleaved to give D-glyceraldehyde-3-phosphate, finally leading to 2′-deoxyribonucleosides via aldol condensation of D-glyceraldehyde-3-phosphate with acetaldehyde

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and enzymatic introduction of chemically synthesized nucleobases would involve too many reaction steps (Fig.1). Therefore, a strategy involving only few reaction steps using enzyme preparations or whole cells as catalysts of the central reaction steps was sought. Two strategies were evaluated.

The first strategy, the straightforward approach of breaking down intra- or extracellular DNA for the production of 2′-deoxyribonucleosides, is not necessarily suitable when it comes to large-scale application (Fig.2). The isolation of genomic DNA from microorganisms is not very effective as only a minor part of the cellular mass consists of DNA [1]. In addition, the RNA content of the cell can exceed the DNA content by far. This makes purification of DNA unattractive. Therefore, the release of DNA into the medium without lysis of the cells would be ideal, and purification procedures could be minimized. *Pseudomonas*, *Arthrobacter*, and *Micrococcus* species are the most prominent DNA-excreting microorganisms described in literature [2]. Although strains of *Corynebacterium ammoniagenes* are known to accumulate

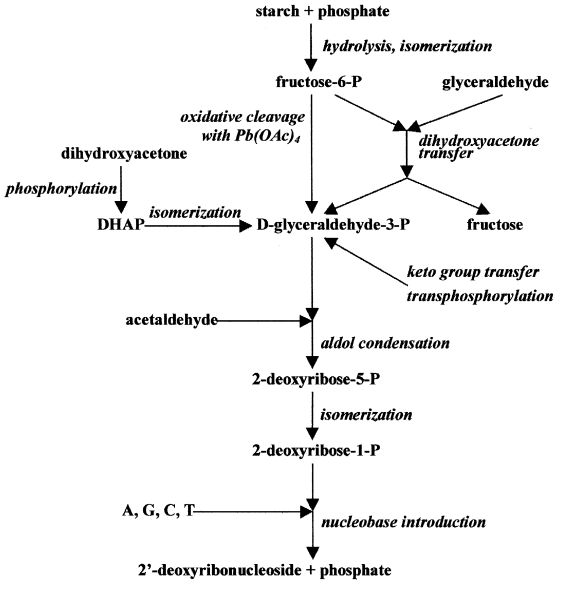

Fig. 1. Complex multistep enzymatic de novo synthesis of 2′-deoxyribonucleosides based on natural biosynthetic pathways.

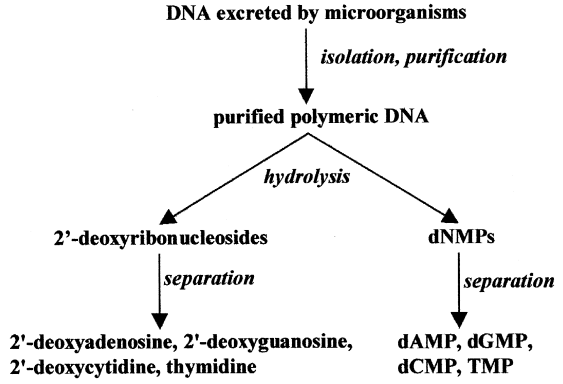

Fig. 2. DNA excretion by microorganisms followed by isolation and hydrolysis of excreted DNA with ensuing separation of the products (first strategy).

ribonucleosides extracellularly, e.g., in the production of flavor enhancers like IMP [3] and GMP [4], they do not seem to be able to excrete 2′-deoxyribonucleosides. The DNA excretion rates reported in literature are too low for commercial production. In addition, extracellular DNA measured in the culture broth of some microorganisms is due to cell lysis [5–9]. Fermentation of halophiles [10] is characterized by low cell yields and therefore suboptimal.

The central step in the approach of the second strategy is the 2′-reduction of the ribose moiety (Fig.3). Ribonucleotide reductases are

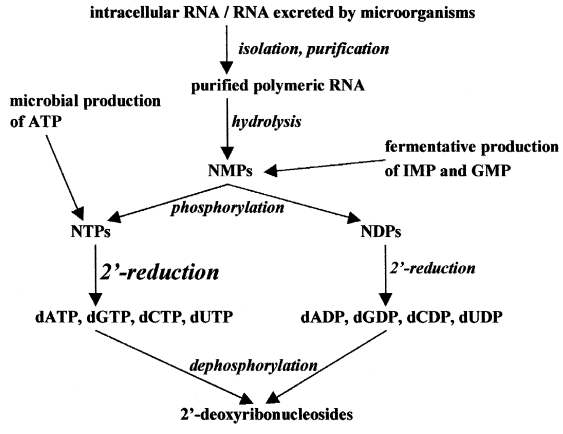

Fig. 3. Production of ribonucleoside-5′-monophosphates (NMPs) by degradation of RNA or by fermentation, followed by 2′-reduction of the ribose moiety of further phosphorylated NMPs and separation of the reaction products (second strategy).

key enzymes as they are the main regulators of 2′-deoxyribonucleotide synthesis in the cells. They reduce the C-2 of the ribose moiety of ribonucleoside diphosphates or ribonucleoside triphosphates depending on the type of ribonucleotide reductase utilized. Extraction and hydrolysis of yeast RNA is of industrial importance in the production process of flavor enhancers like IMP and GMP. The industrial production of IMP and GMP is in the order of kilotons per year. The most convenient way to access RNA on a large-scale is cultivation of *Candida utilis* or *Saccharomyces cerevisiae* followed by extraction of RNA [11,12]. The fermentative production with bacterial mutants is the most important source of nucleosides [3,4,13]. A release from feedback regulation working in the de novo nucleotide biosynthesis, lack of nucleotide degrading activities, and removal of the membrane permeability barrier for nucleotide excretion are prerequisites for the effective production of nucleosides. Screening for overproducing mutants has been extensively performed. Several strains from the genera *Bacillus*, *Corynebacterium*, and *Arthrobacter* are used commercially. ATP can be produced via phosphorylation of adenosine or AMP [14].

After having decided to pursue the second strategy (see above) the focus was given to the reduction step. The 5′-deoxyadenosylcobalamin (AdoCbl) dependent ribonucleosidetriphosphate reductase (RTPR) of *Lactobacillus leichmannii* was thought to be the most suitable enzyme for preparative reduction reactions due to its monomeric structure, an available recombinant source, and the well studied reaction mechanism. The enzyme has one binding site for NTP substrates, a separate binding site for allosteric effectors (2′-deoxyribonucleoside-5′-triphosphates, dNTPs), and a binding site for the AdoCbl [15–17]. Five cysteine residues of RTPR are involved in the reduction of ribonucleoside-5′-triphosphates (NTPs) [18]. Binding of appropriate dNTP effectors enhances the substrate turnover [19]. AdoCbl functions as the radical chain initiator [20,21].

2. Experimental

2.1. Microorganisms and chemicals

E. coli HB101 pSQUIRE was a gift of Prof. JoAnne Stubbe, MIT, Cambridge, MA, USA. ITP and alkaline phosphatase (from bovine intestinal mucosa) were from Sigma. All other chemicals were bought from Fluka.

2.2. Cultivation of microorganisms

E. coli HB101 pSQUIRE from −80°C stock cultures was streaked onto LB + Amp agar plates (LB medium supplemented with 50 μg/ml ampicillin) and incubated for 24 h at 37°C. One-hundred-milliliter conical flasks containing 50 ml LB + Amp medium were inoculated with single colonies from agar plates and incubated at 37°C shaking with 220 rpm for 24 h (HT labshaker/Infors, Binningen, Switzerland). A 30-l bioreactor (MBR Bioreaktor, Wetzikon, Switzerland) containing 20 l of LB + Amp medium was inoculated with 300 ml of shake flask preculture. The fermenter was operated for 24 h at 37°C, with mixing at 500 rpm, with an air inflow of 10 l/min. Cells were harvested by continuous flow centrifugation ($18\,000 \times g$, 150 ml/min, 4°C, TZ-28 rotor, RC-5B centrifuge/Kendro Laboratory Products, Newtown, CT, USA). The obtained cell paste was stored at −80°C.

2.3. Purification of RTPR

Frozen cells of *E. coli* HB101 pSQUIRE were thawed and a 40% (w/v) suspension in 67 mM potassium phosphate buffer (KPi) pH 8.0 was prepared. The cells were disrupted by sonication (Sonopuls HD2070, SH213G Booster Horn, TT13 plate, 35 min on ice, 10% duty cycle, 36% power output/Bandelin Electronic, Berlin, Germany) and centrifuged ($15\,000 \times g$, 20 min, 4°C, Sorvall SS-34 rotor). Crystalline ammonium sulfate was added to the supernatant to 20% saturation. After incubation on ice for

120 min and centrifugation (14 000 × *g*, 30 min, 4°C, Sorvall SS-34 rotor) ammonium sulfate was added to the supernatant to 55% saturation. Following incubation on ice for 120 min and centrifugation (14 000 × *g*, 30 min, 4°C, Sorvall SS-34 rotor), the resulting pellet was resuspended in 67 mM KPi pH 7.0. After desalting (DG-10 column/Bio-Rad Laboratories, Richmond, CA, USA) and sterile filtration (0.45 μm) the sample was loaded onto a hydroxyapatite column (Bio-Rad MacroPrep Ceramic Hydroxyapatite Type I, 20 μm bead size). Elution and separation of RTPR from the majority of contaminant proteins was achieved by applying an isocratic flow of 30 mM KPi pH 7.0 to the column. Active fractions were pooled and concentrated (YM-30 membrane, Amicon chamber/Amicon., Beverly, MA, USA). The concentrated RTPR preparation was stored at −20°C.

2.4. Determination of protein content

The protein content of RTPR preparations was determined according to Bradford [22] using Bio-Rad protein assay dye reagent concentrate. A bovine serum albumin standard curve served to calibrate the measurements.

2.5. Enzyme assays

If not stated differently in the text, enzyme assays were incubated at 25°C in nontransparent microtubes with shaking at 700 rpm (Thermomixer 5436/Eppendorf-Netheler-Hinz, Hamburg, Germany). The RTPR utilized for the determination of kinetic parameters was from the concentrated hydroxyapatite chromatography preparation.

2.6. Standard enzyme assays

Standard enzyme assays were performed to determine the activity of RTPR in samples from purification steps and preparations used for kinetic experiments. One unit of RTPR reduced 1 μmol of ITP per minute under the given reaction conditions. The assays contained: sodium acetate 1 M, AdoCbl 100 μM, ITP 10 mM, 1,4-dithio-DL-threitol (DTT) 100 mM, RTPR preparation. All components were dissolved in 100 mM Tris buffer pH 8.3. NTP stock solutions were adjusted to pH 8.3 with NaOH 2 M prior to use in enzyme assays. Enzyme assays were performed in nontransparent microtubes with shaking at 700 rpm at 25°C.

2.7. Processing of enzyme assays

Samples of the enzyme assays were heated to 95°C for 10 min, then centrifuged for 4 min at 18 000 × *g* (Biofuge 15/Heraeus Sepatech GmbH, Osterode, Germany). Twenty microliters of the sample was added to 200 μl of phosphatase buffer (glycylglycine 150 mM, $ZnCl_2$ 50 mM, alkaline phosphatase 40 U/ml, pH 7.9) and incubated for 120 min at 30°C. After heating to 95°C for 10 min the ribonucleoside/2′-deoxyribonucleoside content was determined by HPLC (Merck LiChrospher 125-4 RP-18, 1 ml/min, detection at 254 nm, 97% 10 mM KPi pH 2.0 + 3% methanol for separation of inosine/2′-deoxyinosine, adenosine/2′-deoxyadenosine or guanosine/2′-deoxyguanosine, 100% 10 mM KPi pH 2.0 for separation of cytidine/2′-deoxycytidine or uridine/2′-deoxyuridine).

2.8. Enzyme kinetics

In each of the experiments only one reaction parameter was varied. Model equations deduced from simple Michaelis–Menten kinetics were developed and tested for fit to the experimental data from enzyme assays. The kinetic data of RTPR with respect to affinity of NTP substrates, the cofactor AdoCbl, and the reducing agent DTT were determined. A model equation characterizing the reaction kinetics of RTPR was assembled from data of several experiments.

2.9. Preparative reduction reactions

All reactions were performed in nontransparent vessels. The reaction conditions and initial assay composition for the 1-g reductions of ITP, ATP, GTP and UTP were as follows: 25°C, pH 8.2, shaking at 120 rpm, incubation time 360 min, total reaction volume 80 ml, RTPR 80 U, AdoCbl 0.053 g, NTP 1 g (purity of substrates: ITP ~97%, ATP ~97%, GTP >90%, UTP ~90%), sodium acetate 1 M, DTT 1.246 g. Two volumes of cold ethanol were added to one volume of reaction mixture to stop the reaction and precipitate the reaction product [23]. After incubation on ice for 30 min the resulting precipitate was lyophilized.

3. Results

3.1. Purification of RTPR from E. coli HB101 pSQUIRE

Recombinant *L. leichmannii* RTPR [24] was used in the study presented here since the expression level of RTPR in *L. leichmannii* DSM 20355 was found to be too low for use in preparative reduction reactions. Resuspended cells of *E. coli* HB101 pSQUIRE were sonicated, centrifuged, and subjected to ammonium sulfate precipitations. After desalting and sterile filtration, the RTPR preparation was further purified on a hydroxyapatite column. Active fractions were pooled and concentrated by ultrafiltration. The overall yield was 60.2%. Roughly 34 U of RTPR with a specific activity of 0.61 U/mg could be purified from 1 g *E. coli* HB101 pSQUIRE cells (wet weight).

3.2. Allosteric effectors

High concentrations of sodium formate, potassium formate, sodium acetate, sodium propionate, ammonium formate, ammonium acetate, and dipotassium phosphate in the reaction mixture enhanced the reaction rate substantially, making the addition of allosteric effectors (dNTPs) of RTPR superfluous. The peak reduc-

Table 1
Influence of salt concentration on RTPR activity in enzyme assays using ITP as substrate
Numbers below compound names indicate RTPR activity under indicated salt concentrations compared to activity in reactions without addition of salt. Reaction conditions and initial concentrations: pH 8.3, 25°C, salt 0–2 M, ITP 10 mM, AdoCbl 100 μM, DTT 100 mM, RTPR.

Concentration [M]	Ammonium formate	Sodium formate	Potassium formate	Sodium acetate
0	1.0	1.0	1.0	1.0
0.3	2.9	3.6	4.2	4.6
0.6	3.5	5.9	7.4	10.1
0.8	3.6	7.1	9.3	12.9
1.0	3.6	7.9	10.5	14.6
1.2	3.6	9.0	11.8	15.6
1.4	3.8	9.0	12.3	14.9
1.7	3.7	8.7	11.4	12.4
2.0	3.6	7.4	10.5	9.3

Concentration [M]	Sodium chloride	Sodium propionate	Ammonium acetate	Dipotassium phosphate
0	1.0	1.0	1.0	1.0
0.3	1.7	5.1	4.1	12.5
0.6	0.8	8.8	7.4	18.1
0.8	0.5	10.0	9.0	17.4
1.0	0.3	10.3	10.5	15.1
1.2	0.1	9.3	11.8	12.7
1.4	0.0	8.2	12.8	9.7
1.7	0.0	6.0	13.0	1.5
2.0	0.0	3.8	12.1	0.3

Table 2
Artificial reducing agents for RTPR active center recycling
Effectiveness was measured compared to effectiveness of DTT (100%). Reaction conditions and initial concentrations: pH 8.3, 25°C, sodium acetate 1 M, reducing agent 60 mM, ITP 10 mM, AdoCbl 100 μM, RTPR. Reduction of ITP served as measure of effectiveness of reducing agents.

Reducing agent	Effectiveness [%]
1,3-Propanedithiol	52.3
1,4-Butanedithiol	14.6
1,5-Pentanedithiol	0.0
1,4-Dithio-DL-threitol (DTT)	100.0
1,4-Dithioerythritol (DTE)	99.9
2,3-Dimercapto-1-propanol	3.5
2-Dimethylaminoethanethiol HCl	2.2
2-Mercaptoethanol	0.0
Cysteamine	0.0
L-Glutathione, reduced	0.0
meso-2,3-Dimercaptosuccinic acid	0.0
N-(2-Mercaptopropionyl)-glycine	0.0
Na-(2-Mercaptoethane)-sulfonate	3.0
Bis(2-mercaptoethyl)sulfide	0.0
Tris(2-carboxyethyl)phosphine HCl	0.0
Tris(2-cyanoethyl)phosphine	0.0
Na-(3-mercaptopropane)sulfonate	0.0
DL-2,3-Dimercaptopropane-sulfonate	3.0
Bis(2-mercaptoethyl)sulfone[a]	60.6

[a] Not completely soluble at 60 mM concentration.

tion rate with an increase of more than 15 times compared to RTPR enzyme assays without salt addition was recorded when sodium acetate (1.0–1.4 M) or dipotassium phosphate (0.6–1.0 M) were added to the reaction (Table 1). Sodium chloride was not effective.

3.3. Artificial reducing agents for RTPR active center recycling

We identified several artificial reducing agents suitable for re-reduction of RTPR active center Cys residues. DTT followed by 1,4-dithioerythritol (DTE), bis-(2-mercaptoethyl)-sulfone, and 1,3-propanedithiol were the most effective reducing agents under the reaction conditions applied (Table 2). The solubility of bis-(2-mercaptoethyl)-sulfone in the reaction mixture was only about 12 mM. The Michaelis constants of DTT and 1,3-propanedithiol were 16.6 and 7.1 mM, respectively.

3.4. Reaction kinetics

We determined the activity of RTPR towards the substrates ATP, CTP, GTP, ITP, and UTP. A comparable reaction velocity with purine triphosphates (ATP, GTP, ITP) was observed. The RTPR had a lower reaction velocity with the pyrimidine triphosphate substrates (CTP, UTP) tested (Fig.4). DTT has only a relatively short half-life time under the reaction conditions applied (pH 8.3, high salt). The decay of DTT has led to incomplete substrate turnover as can be seen in Fig.4. Michaelis constants (K_M) of RTPR for the different NTP substrates, AdoCbl, and DTT were measured. The following values were obtained: $K_{M_{ATP}} = 4.8$ mM, $K_{M_{CTP}} = 1.4$ mM, $K_{M_{GTP}} = 2.9$ mM, $K_{M_{ITP}} = 4.6$ mM, $K_{M_{UTP}} = 3.0$ mM, $K_{M_{AdoCbl}} = 31$ μM, and $K_{M_{DTT}} = 16.6$ mM. Substrate inhibition was not observed with any of the substrates of RTPR under the employed reaction conditions. A simulation of the reaction kinetics of RTPR was developed by integration of the relevant reaction parameters into one model formula (Eq. (1)). The time course of the 2′-reduction of NTPs by RTPR could be calculated with this formula composed of simple Michaelis–Menten type kinetics of

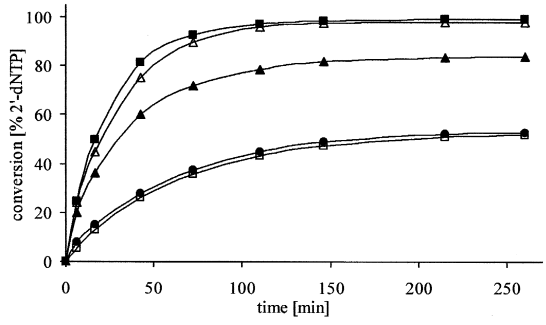

Fig. 4. Time course of substrate conversion by RTPR. Reaction conditions and initial assay composition: pH 8.3, 25°C, sodium acetate 1 M, NTP 50 mM (ATP, CTP, GTP, ITP, UTP), AdoCbl 300 μM, DTT 150 mM, RTPR 1.6 U/ml. (–▲–) dATP [% 2′-reduction], (–●–) dCTP [% 2′-reduction], (–△–) dGTP [% 2′-reduction], (–■–) dITP [% 2′-reduction], (–□–) dUTP [% 2′-reduction].

the reducing agent DTT, the coenzyme AdoCbl, and a NTP substrate.

$$V = \frac{V_{max_{RTPR,NTP}}[\text{NTP}][\text{AdoCbl}][\text{DTT}]}{(K_{M_{NTP}} + [\text{NTP}])(K_{M_{AdoCbl}} + [AdoCbl])(K_{M_{DTT}} + [\text{DTT}])} \quad (1)$$

Compounds in rectangular brackets denote concentrations.

3.5. Characterization of RTPR

The RTPR activity contained in a desalted sample of the resuspended pellet from 55% ammonium sulfate precipitation showed to have a half-life time of 21 to 23 days when incubated at 23°C in the dark. The highest activity of RTPR was found to be between pH 7.5 and 8.5, with an optimum around pH 8.2 (Fig.5).

3.6. Preparative reductions

The observed conversion rates of 1-g 2′-reductions of ITP, ATP, GTP and UTP were 96.5% (dITP), 97.5% (dATP), 91.8% (dGTP) and 90.8% (dUTP), respectively. These conversion rates correspond to full conversion of the NTP substrates taking into account the purities of the utilized substrates. The time course of the reactions was in accordance with the kinetic model calculation presented earlier in the text (Eq. (1)).

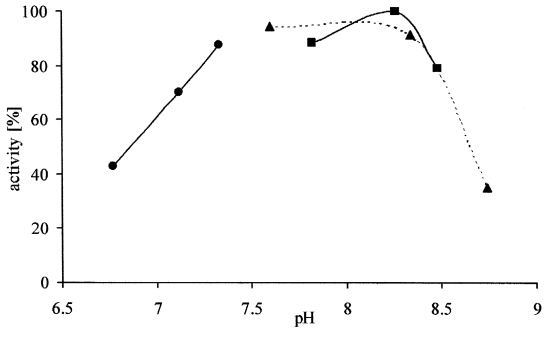

Fig. 5. pH dependence of RTPR activity. Reaction conditions and initial assay composition: 25°C, sodium acetate 1 M, ITP 10 mM, AdoCbl 100 μM, DTT 100 mM, RTPR 0.05 U/ml. (–●–) KPi buffer 100 mM, (–■–) Tris buffer 100 mM, (- -▲- -)glycine buffer 100 mM.

4. Discussion

We believe the 2′-reduction of NTPs investigated in this report to be a key step in the preparative enzymatic synthesis of 2′-deoxyribonucleosides. The production of 2′-deoxyribonucleosides from salmon milt is dependent on the output of the fisheries industry and can not respond to sudden high demands of the market whereas the enzymatic approach is mainly dependent on the scale-up of the fermentation capacity available to produce RTPR. We have shown that the use of recombinant *L. leichmannii* RTPR as biocatalyst in the preparative synthesis of 2′-deoxynucleoside-5′-triphosphates is a suitable strategy. The data of the substrate-specificity experiments and the gram-scale reduction reactions presented in this report show that 2′-reduction of ITP, ATP, GTP, CTP, and UTP by RTPR are reactions showing similar turnover kinetics with the pyrimidine NTPs having a somewhat lower reaction velocity. This is in accordance with observations made by other investigators [25]. Having a half-life of over 20 days at room temperature, RTPR is an enzyme suitable for application in preparative synthesis. All tested NTP substrates had K_M values below five millimolar concentration and did not show remarkable inhibition on RTPR activity under the special reaction conditions applied, allowing the performance of batch reactions with small reaction volumes and high substrate concentrations. Some differences can be found when our kinetic data of RTPR is compared with the data presented by other authors [21]. This is partly due to the lower reaction temperatures chosen to stabilize the reacting components at the slightly alkaline conditions, especially the reducing agents and NTP substrates. The relatively quick decay of DTT under the employed reaction conditions (half life DTT < 2 h) calls for repetitive addition of

reducing agent in the course of the reaction process. Decomposition of NTP substrates in the reaction was minor but NTPs cannot be stored without decomposition at alkaline pH [26]. Another factor that influenced the kinetic behaviour of RTPR was the high concentration of salt present in our experiments. Due to high salt concentration the addition of allosteric effectors (dNTPs) was superfluous, altering also the kinetic behavior of RTPR compared to natural conditions [17]. Substrate affinity changes elicited by dNTP reaction products affecting the selectivity of RTPR via the allosteric effector binding site which is normally observed under natural conditions [21] was surprisingly lost in reactions containing high concentrations of salt. The possibility of precipitating dNTPs by addition of cold ethanol at the endpoint of the reaction facilitates the isolation of relatively pure dNTP products out of the reaction mixtures [25]. The scale-up of the ATP reduction to 100 g is the next step envisaged. Until now, ATP is the only ribonucleoside-5′-triphosphate commercially available on larger scale. Therefore, the chemical and enzymatic synthesis of NTPs needs to be investigated as well in order to provide a supply of NTPs for 2′-reduction by RTPR with reasonable costs.

Acknowledgements

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