Biosci. Biotechnol. Biochem., **64** (9), 1836–1841, 2000

Preparative 2′-Reduction of ATP Catalyzed by Ribonucleotide Reductase Purified by Liquid-Liquid Extraction

André BRUNELLA,[1] Miguel ABRANTES,[2] and Oreste GHISALBA[3]

[1]*Outcomes International, Malzgasse 9, CH-4052 Basel, Switzerland*
[2]*Logoplaste SA, P-1000 Lisboa, Portugal*
[3]*Novartis Pharma AG, WSJ-508.202A, CH-4002 Basel, Switzerland*

Received February 7, 2000; Accepted April 27, 2000

Recombinant *Lactobacillus leichmannii* ribonucleosidetriphosphate reductase (RTPR, E.C.1.17.4.2) constitutively expressed by *E. coli* HB101 pSQUIRE has been purified from sonicated cell material in a one-step procedure by PEG 4000 (16% (w/w))/phosphate (7% (w/w)) liquid-liquid extraction. A high yield of 75.1% RTPR in the top phase and a partitioning of 8.5:1 between total RTPR activity in top and bottom phase were obtained in this preparative system. The RTPR-containing top phase was used to reduce ATP in the 2′-position on a gram scale with high final conversion and yield proving the ribonucleotide reductase approach feasible for the preparative synthesis of 2′-deoxyribonucleotides. High concentrations of sodium acetate in the reaction served to substitute for allosteric effectors of RTPR. 1,4-Dithio-DL-threitol was used as an artificial reducing agent for RTPR.

Key words: liquid-liquid extraction; enzymatic synthesis; ribonucleotide reductase; ATP; 2′-deoxyadenosine-5′-triphosphate

The application of ribonucleoside triphosphate reductase (RTPR, E.C.1.17.4.2) for preparative synthesis reactions is a challenge since the enzyme is subject to complex allosteric regulation,[1] has a cofactor requirement,[2] and needs a second substrate that recycles active center thiols after each round of substrate reduction.[3] We have replaced allosteric effectors (2′-deoxyribonucleoside triphosphates) by high concentrations of sodium acetate and used 1,4-dithio-DL-threitol as an artificial reducing agent for RTPR.[4] Additional to addressing issues of reaction engineering, we have seen that establishment of a simple and cheap purification method is crucial for the successful application of RTPR as a biocatalyst on a large scale. Proteins, nucleic acids, or cell debris can be partitioned into the top and bottom phases of aqueous two-phase systems. The partition behavior of biopolymers in aqueous two-phase systems can be affected by a number of factors *e.g.*, type of polymers, molecular weight and distribution of polymers, length of a tie-line, type and concentration of added salts, pH, and temperature.[5] Besides using liquid-liquid extraction to clear away cell debris, many enzymes were actually purified with this method.[6] Due to the mentioned advantages, the purification of RTPR from sonicated cell material with liquid-liquid extraction was envisaged and investigated.

Materials and Methods

Microorganisms. E. coli HB101 pSQUIRE[7] was a gift of Prof. JoAnne Stubbe, MIT, Cambridge MA, USA. *E. coli* HB101 pSQUIRE was cultivated as described.[4]

Cell disruption. Twenty-five grams of frozen cells (−80°C) of *E. coli* HB101 pSQUIRE were thawed and a 40% (w/w) suspension in Tris buffer 100 mM pH 8.0 was prepared. Sonication of the cells lasted 12 min (power output 45%, Sonopuls HD2070 sonicator with SH213G booster horn and TT13 plate/Bandelin Electronic GmbH, Berlin, Germany).

Ammonium sulfate precipitations. Crystalline ammonium sulfate was added to the desired saturation to the supernatant of a sonicated cell suspension that had been freed from cell debris by centrifugation (12000 rpm, 4°C, 30 min, Sorvall GSA rotor). After incubation on ice for 120 min, the preparation was centrifuged and either the supernatant or the pellet, depending on the location of RTPR, was used for further purification.

Evaluation of PEG/ammonium sulfate liquid-liquid extraction systems. The pellet of a 55% ammonium sulfate precipitation of *E. coli* HB101 pSQUIRE sonication supernatant was resuspended to 25% (w/w) in a 10% saturated ammonium sulfate solu-

To whom correspondence should be addressed. Oreste GHISALBA, E-mail: oreste.ghisalba@pharma.novartis.com; Phone: +41-61-324-3084; Fax: +41-61-324-2103

tion. Accurate amounts of PEG and crystalline ammonium sulfate were diluted in the resuspended pellet. Phase separation after vigorous vortexing was done by centrifugation (14000 rpm, 3 min, Biofuge 15/Heraeus Sepatech, Osterode, Germany). Volumes, protein concentrations, and RTPR activities of top and bottom phase (and of the interphase pellet where present and extractable) were measured in case a two-phase system had formed after centrifugation.

Preparative PEG/ammonium sulfate liquid-liquid extraction. Following a 20% ammonium sulfate precipitation in the sonication supernatant of 25 g of *E. coli* HB101 pSQUIRE cells and removal of the formed pellet, ammonium sulfate was added to the supernatant until a saturation of 55% was reached. The pellet of this precipitation was resuspended to 25% (w/w) in Tris buffer 100 mM pH 8.0 supplied with 5.5% (w/w) ammonium sulfate. Then, solid polyethylene glycol 4000 (PEG 4000) and additional ammonium sulfate were dissolved until concentrations of 11% (w/w) and 9% (w/w) were reached, respectively. After centrifugation (10000 rpm, 4°C, 10 min, Sorvall SS-34 rotor) the top phase was recovered and the pH adjusted to 8.0. The major part of RTPR accumulated in this top phase.

Evaluation of PEG/phosphate liquid-liquid extraction systems. The evaluation of PEG/phosphate liquid-liquid extraction systems was straight-forward. The phosphates consisted of K_2HPO_4 and KH_2PO_4 mixed in the ratio 6.5:1.0 (w/w) corresponding to a buffer of around pH 7.5. PEG and phosphates were used to dilute sonicated *E. coli* HB101 pSQUIRE cell suspensions of varying concentration (10%–40% (w/w)). Volumes, protein concentrations, and RTPR activities of top and bottom phase were measured after centrifugation (14000 rpm, 3 min, Biofuge 15).

Preparative PEG/phosphate liquid-liquid extraction. The sonication supernatant of 25 g *E. coli* HB101 pSQUIRE cells was partitioned in PEG 4000/phosphate liquid-liquid extraction. 16% (w/w) PEG 4000 and 7% (w/w) phosphates were dissolved in sonicated 40% (w/w) cell suspension. The phosphates consisted of K_2HPO_4 and KH_2PO_4 mixed in the ratio 6.5:1.0 (w/w). After an ensuing centrifugation (15000 rpm, 4°C, 10 min, Sorvall SS-34 rotor), the clear top phase containing most of the RTPR activity was separated from the bottom phase where cell debris and a considerable amount of protein were located.

Preparative reduction of ATP. All preparative reduction reactions were done in nontransparent vessels incubated at 25°C shaking with 100 rpm on a rotary shaker. RTPR was from the top phase of PEG 4000 (16% (w/w))/phosphate (7% (w/w)) liquid-liquid extraction. Reactions were done in the following buffers: Tris 100 mM pH 8.3 + sodium acetate 1 M (Exp. 1–5), Tris 100 mM pH 8.0 + sodium acetate 1 M + EDTA 2 mM (Exp. 6). Concentrations of ATP, RTPR, and AdoCbl can be found in Table 5. Compared to ATP, a three-fold molar excess of DTT was initially added to the reactions (Exp. 6: four-fold excess of DTT). The same amount of DTT was resupplied to the reactions after 60 min (Exp. 6: 50 min) of incubation. Reactions were stopped after 120 min (Exp. 6: 100 min). Two volumes of dry ice-cooled ethanol were added to one volume of reaction mixture at the endpoint of the reaction time to stop the reaction and precipitate the reaction product. After incubation on dry ice for 120 min and centrifugation (8000 rpm, 4°C, 30 min, Sorvall GSA rotor), the resulting precipitate was diluted in deionized water and lyophilized.

Measurement of protein. The protein content was measured by the method of Bradford[8] using Bio-Rad protein assay dye reagent concentrate. A bovine serum albumin standard curve served to calibrate the measurements.

Enzyme assays. One unit of RTPR reduced 1 μmol of ATP per minute under the given reaction conditions. Enzyme assays were incubated at 25°C in nontransparent microtubes shaken at 700 rpm (Thermomixer 5436/Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The assays contained: sodium acetate 1 M, AdoCbl 100 μM, ATP 40 mM, 1,4-dithio-DL-threitol (DTT) 100 mM, RTPR preparation. All components were dissolved in 100 mM Tris buffer pH 8.3. The ATP stock solutions were adjusted to pH 8.3 with NaOH 2 M before use in enzyme assays. Enzyme assays were stopped by heating to 95°C for 10 min. After centrifugation (14000 rpm, 4 min, Biofuge 15), 20 μl of the supernatant was added to 200 μl of phosphatase buffer (glycylglycine 150 mM, $ZnCl_2$ 50 mM, alkaline phosphatase (from bovine intestinal mucosa, Sigma) 40 U/ml, pH 7.9) and incubated for 120 min at 30°C. After heating to 95°C for 10 min the adenosine/2′-deoxyadenosine content was measured by HPLC (Merck LiChrospher 125-4 RP-18, 1 ml/min, detection at 254 nm, isocratic run: 97% KPi pH 2.0 10 mM + 3% methanol; retention times: adenosine 4.5 min/2′-deoxyadenosine 5.6 min).

Results

Evaluation of PEG/ammonium sulfate liquid-liquid extraction systems

PEG 400, PEG 1000, PEG 4000, and PEG 8000 were evaluated as components of liquid-liquid extrac-

tion systems for RTPR. Four different PEG/ammonium sulfate liquid-liquid extraction systems were tested for each PEG species. In the presence of resuspended pellet from a 55% ammonium sulfate precipitation, all pairs of PEG/ammonium sulfate tested gave two-phase systems due to displacement of the binodal curve elicited by the cell material. Almost all systems tested led to formation of pellets between top and bottom phases resulting from precipitation of proteins caused by ammonium sulfate. PEG 400 and PEG 8000 systems did not increase the purity of RTPR since the majority of RTPR activity accumulated in the same phase as the bulk of protein. The most promising results were recorded for PEG 1000 and PEG 4000 (Table 1). The PEG 4000 (11% (w/w))/ammonium sulfate (9% (w/w)) system was finally selected for preparative experiments because it had a good partitioning of RTPR into the top phase and additionally a large part of other proteins were accumulated in the bottom phase. The preparative data listed in Table 2 showed that liquid-liquid extraction with a PEG 4000 (11% (w/w))/ammonium sulfate (9% (w/w)) system led to accumulation of RTPR and a considerable increase of specific activity in the top phase. Unfortunately, yield in the top phase was suboptimal (41.7%). This was mainly due to inactivated RTPR lost in the interphase pellet.

Evaluation of PEG/phosphate liquid-liquid extraction systems

Two PEG/phosphate systems were tested for the purification of RTPR: PEG 1000 (18% (w/w))/phosphate (7% (w/w)) and PEG 4000 (16% (w/w))/phosphate (7% (w/w)). The systems are below the binodals in the concentration diagram *i.e.*, in the one-phase area. Nevertheless, two-phase systems were expected in the presence of cell debris due to displacement of the binodal curve by the cell material. Within the whole range of concentration of sonicated cell material tested (10%–40% (w/w)) no two-phase systems formed with the PEG 1000 (18% (w/w))/phosphate (7% (w/w)) systems. The systems containing PEG 4000 worked well. The major part of the RTPR activity was found in the top phase, while cell debris and a considerable share of cell protein was sorted to the bottom phase. Similar protein partition was recorded for all experimental conditions (Table 3). The RTPR activity partition coefficient of 17.5 recorded in the 20% crude extract experiment is exceptional. Anyhow, the partition of activity was good at all concentrations of crude extract, and therefore, it was decided to minimize the necessary extraction volume and use 40% (w/w) crude extract preparations in preparative scale extractions.

Table 1. Experimental Data of the Most Suitable PEG/Ammonium Sulfate Systems (AMS) for Purification of RTPR

PEG [MW]	PEG [% w/w]	AMS [% w/w]	Phase	Interface pellet size	Activity [U]	Protein [mg]	Partition of RTPR activity	Partition of protein
			top		23.6	23.1		
1000	15	14	bottom		0.0	5.3	>100	4.36
			pellet	large	1.1	11.1		
			top		20.2	19.9		
1000	16	15	bottom		0.0	3.9	>100	5.10
			pellet	large	2.9	16.5		
			top		13.0	7.8		
4000	10	8	bottom		10.5	34.7	1.2	0.22
			pellet	small	n.d.	n.d.		
			top		18.1	11.2		
4000	11	9	bottom		2.3	30.5	7.9	0.37
			pellet	medium	0.2	3.8		
			top		18.2	10.5		
4000	12	10	bottom		0.7	27.1	26.0	0.39
			pellet	medium	1.5	7.1		

Interphase pellets were resuspended. Partition values are indicated as ratio of total top phase RTPR activity or total top phase protein and total bottom phase RTPR activity or total bottom phase protein, respectively.

Table 2. RTPR Purification Involving PEG 4000 (11% (w/w))/Ammonium Sulfate (9% (w/w)) Liquid-liquid Extraction

Fraction	Volume [mL]	Protein [mg/mL]	Activity [U/mL]	Protein [mg]	Activity [U]	Spec. act. [U/mg]	Recovery [%]
Supernatant sonication	51	62.1	22.2	3167	1132	0.36	100.0
Supernatant 20% AMS precipitation	48	58.0	19.6	2784	941	0.34	83.1
Resuspended pellet 55% AMS precip.	54	34.7	15.6	1874	842	0.45	74.4
Top phase PEG 4000/ammonium sulfate	16	28.4	29.5	454	472	1.04	41.7
Bottom phase PEG 4000/$(NH_4)_2SO_4$	39	25.0	2.0	975	79	0.08	7.0

Preparative PEG/phosphate liquid-liquid extraction

Table 4 summarizes purification of RTPR by the PEG 4000 (16% (w/w))/phosphate (7% (w/w)) two-phase system. Liquid-liquid extraction with this system proved to be very effective. The procedure is simple and fast, RTPR yield and specific activity in the top phase are high, and the scale of the whole procedure can be easily enlarged. The PEG-rich top phase was a clear amber colored liquid after centrifugation, and all cell debris and a considerable part of proteins were partitioned to the phosphate rich bottom phase.

Preparative reduction of ATP

A number of ATP reduction experiments with RTPR preparation from the top phase of preparative PEG 4000 (16% (w/w))/phosphate (7% (w/w)) liquid-liquid extraction were done. High initial concentrations of ATP in the reactions negatively influenced final conversion rates but positively affected product recovery from ethanol precipitation (Table 5, Exp. 1–3). Although the assays were incubated for 120 minutes and the reactions were resupplied with DTT after 60 minutes of reaction time, full 2′-reduction of ATP was not observed. Even addition of an excess of RTPR as in the 10 g reduction experiments did not increase the conversion rate to over 88% (Table 5, Exp. 4–5). The achieved conversion rates were not fully satisfactory. We mainly attributed the failure of full reduction of ATP in the reactions to rapid decay of DTT, phosphatases in the RTPR preparation, and partially to substrate inhibition when high initial concentrations of ATP were chosen. Just adding more DTT to the reactions did

Table 3. Evaluation of RTPR Purification by PEG 4000/Phosphate Systems

Crude extract [% w/w]	Phase	Total activity [U]	Total protein [mg]	Partition of activity	Partition of protein
10	top	2.59	3.68	7.2	0.53
	bottom	0.36	6.95		
15	top	4.52	5.20	5.9	0.58
	bottom	0.76	8.94		
20	top	11.89	6.48	17.5	0.60
	bottom	0.68	10.82		
25	top	8.77	7.49	5.3	0.63
	bottom	1.64	11.86		
30	top	14.56	8.77	3.9	0.67
	bottom	3.73	13.07		
35	top	14.65	9.34	6.0	0.72
	bottom	2.45	13.01		
40	top	17.30	11.20	4.1	0.67
	bottom	4.25	16.80		

Partition values are indicated as ratio of total top phase RTPR activity or total top phase protein and total bottom phase RTPR activity or total bottom phase protein, respectively.

Table 4. RTPR Purification Involving PEG 4000 (16% (w/w))/Phosphate (7% (w/w)) Liquid-liquid Extraction

Fraction	Volume [mL]	Protein [mg/mL]	Activity [U/mL]	Protein [mg]	Activity [U]	Spec. act. [U/mg]	Recovery [%]
Sonicated cells (suspension)	61	65.3	18.0	3983	1098	0.28	100.0
Top phase PEG 4000/phosphate	38	16.9	21.7	642	825	1.28	75.1
Bottom phase PEG 4000/phosphate	26	102.0	3.7	2652	97	0.04	8.8

Table 5. Overview of Preparative ATP Reduction Experiments

Experiment [No.]	ATP [g]	Experimental conditions			Conversion to dATP [%]	Total recovery [%]
		ATP [mM]	RTPR [U]	AdoCbl [μM]		
1	1	25	30	300	84.8	70.0
2	1	50	30	300	68.0	87.0
3	1	100	30	300	27.9	88.0
4	10	25	750	100	87.5	55.4
5	10	50	600	100	71.8	89.0
6	1	20	120	100	96.5	85.7

The extent of 2′-reduction (conversion to dATP) was measured in the lyophilizate of the isolated product. The total recovery denotes the percentage of initial substrate contained in the lyophilizate from ethanol precipitation, consisting of reduced and unreduced nucleotides (different degree of 5′-phosphorylation).

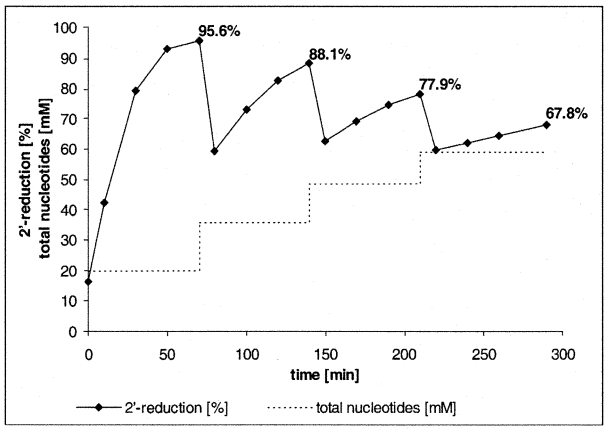

Fig. 1. Consecutive Batch Reduction of ATP in One Pot.
The dashed line denotes the concentration of total nucleotides in the reaction *i.e.*, the sum of the concentrations of 2′-reduced and unreduced ATP, ADP, and AMP. The increase of concentration upon addition of new substrate is not linear due to increase of the total reaction volume.

not have a positive effect on the conversion rates. Replacing the RTPR from liquid-liquid extraction top phase in the one gram ATP reduction reactions by the same amount of RTPR that had additionally been purified by hydroxyapatite chromatography *i.e.*, freed from eventually present phosphatases, did not increase the conversion rate in the reduction reactions. It was decided to add more RTPR to the reaction, adjust the reaction pH to 8.0, and add EDTA to stabilize DTT. The results of this experiment were satisfactory regarding achieved final conversion and yield (Table 5, Exp. 6).

Consecutive batch reduction of ATP

Four consecutive 1-g reduction reactions were done in one pot to test the capacity of the system that has proved to be suitable for the reduction of 1 g of ATP. After 70 min of incubation time of a 1-g ATP batch reduction reaction (as described, Table 5, Exp. 6) 1 g of ATP, 4 molar equivalents of DTT (1.03 g), and 0.84 μmol AdoCbl dissolved in 10 ml of reaction buffer were added to the reaction. The reaction mixture was then incubated for another 70 min. This procedure was repeated two more times. As can be seen from the extent of overall redution in Fig. 1, the RTPR activity in the reaction slowly decreased over time.

Discussion

Within the scope of this project we could show that the use of recombinant RTPR is a suitable strategy to prepare 2′-deoxyribonucleotides on a preparative scale. A growing number of applications for 2′-deoxyribonucleotides as building blocks for antisense oligonucleotides, dNTP substrates for PCR applications, or 2′-deoxynucleoside derivatives as antiviral agents are emerging in the field of biosciences. In our recent publication[4] we have shown that the reduction of all natural NTPs is feasible on a gram scale. However, the preparation of RTPR by hydroxyapatite column chromatography was thought to be too laborious and expensive for application on a larger scale. The PEG 4000 (16% (w/w))/phosphate (7% (w/w)) liquid-liquid extraction system for RTPR presented in this report is fully satisfactory from the point of achieved purification factor and simplicity of application. The best opportunity of partitioning the cell debris into the bottom phase exists in a range along the upper part of the binodal curve but since the cell material also influences the formation of the phase system, phase separation below the binodal curve is possible and done frequently.[9] This was the case in the used PEG 4000 (16% (w/w))/phosphate (7% (w/w)) system where phase separation was observed due to displacement of the binodal line by the added cell material. All cell debris and a considerable part of contaminant proteins could be partitioned to the phosphate containing bottom phase within one step, while the major part of active RTPR was present in the PEG 4000 containing top phase. The achieved partition of 8.5 for total RTPR activity between top and bottom phases is comparable or better than many well studied liquid-liquid extraction processes.[6] Optimization of the reaction conditions for the preparative reduction of ATP was essential in order to achieve a high conversion of ATP to dATP. Lowering the reaction pH from the measured activity optimum of RTPR at pH 8.3 down to pH 8.0 and adding EDTA as thiol stabilizing agent influenced the final conversion rate positively. This is in accordance with Stevens *et al.*[10] who have found that addition of EDTA extends the half-life time of thiols in aqueous solutions substantially. It was necessary to add excess RTPR to the reaction to achieve complete reduction of ATP. This disadvantage was made up by the fact the purification of RTPR by liquid-liquid extraction is a simple one-step procedure that can easily be upscaled. Consecutive batch reactions in one pot are possible but some optimization of the reaction conditions still needs to be done.

Acknowledgments

This project has been funded by the SPP BioTech of the Swiss National Science Foundation and by Novartis Pharma AG, Basel, Switzerland.

References

1) Ludwig, W. and Follmann, H., The specificity of ribonucleoside triphosphate reductase. *Eur. J. Biochem.*, **82**, 393–403 (1978).
2) Licht, S., Gerfen, G. J., and Stubbe, J., Thiyl radicals in ribonucleotide reductases. *Science*, **271**,

477–481 (1996).
3) Booker, S., Licht, S., Broderick, J., and Stubbe, J., Coenzyme B12-dependent ribonucleotide reductase: evidence for the participation of five cysteine residues in ribonucleotide reduction. *Biochemistry*, **33**, 12676–12685 (1994).
4) Brunella, A. and Ghisalba, O., Recombinant *Lactobacillus leichmannii* ribonucleosidetriphosphate reductase as biocatalyst in the preparative synthesis of 2′-deoxyribonucleoside-5′-triphosphates. *J. Mol. Catal. B: Enzymatic*, in press.
5) Albertsson, P. A., "Partition of Cell Particles and Macromolecules" 3rd ed., Wiley, New York (1986).
6) Hustedt, H., Kroner, K. H., Menge, U., and Kula, M.-R., Protein recovery using two-phase systems. *Trends in Biotechnology*, **3**, 139–144 (1985).
7) Booker, S. and Stubbe, J., Cloning, sequencing, and expression of the adenosylcobalamin-dependent ribonucleotide reductase from *Lactobacillus leichmannii*. *Proc. Natl. Acad. Sci. USA*, **90**, 8352–8356 (1993).
8) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
9) Kula, M.-R., Kroner, K. H., and Hustedt, H., Purification of enzymes by liquid-liquid extraction. In "Advances in Biochemical Engineering", 24, ed. Fiechter, A., Springer, Berlin and Heidelberg, pp. 73–118 (1982).
10) Stevens, R., Stevens, L., and Price, N. C., The stabilities of various thiol compounds used in protein purifications. *Biochemical Education*, **11**, 70 (1983).