NUCLEOSIDE DIPHOSPHATE KINASE ACTIVITY ASSOCIATED WITH RIBONUCLEOTIDE REDUCTASE

Ulrika von Döbeln
Medical Nobel Institute, Department of Biochemistry I, Karolinska Institute, Stockholm, Sweden

Received August 10,1976

SUMMARY

Ribonucleotide reductase from Escherichia coli contains nucleoside diphosphate kinase activity even in highly purified preparations. Although the bulk of the kinase activity was removed during the initial steps of the ribonucleotide reductase purification some activity remained in the purest fractions. The nucleoside diphosphate kinase reaction is probably catalyzed by a protein different from ribonucleotide reductase. The tight binding between the two enzymes might reflect an association between them in the living cell.

INTRODUCTION

Deoxyribonucleotides are synthesized via a direct reduction of ribonucleotides to deoxyribonucleotides, which is catalyzed by ribonucleotide reductase (1, 2). Since the reduction takes place at the diphosphate level in Escherichia coli (3), the products have to be phosphorylated to serve as substrates for DNA polymerase. This reaction is catalyzed by nucleoside diphosphate kinase.

Ribonucleotide reductase consists of two subunits, proteins B1 (M. W. 160,000) and B2 (M. W. 78,000) (4, 5). During binding experiments with substrates and ribonucleotide reductase we found that highly purified preparations of both protein B1 and B2 contained nucleoside diphosphate kinase activity (6). Miller and Wells have reported the presence of nucleoside diphosphate kinase activity in preparations of DNA polymerases (7). This means that preparations of the enzymes acting on both sides of the nucleoside diphosphate kinase reaction in the series of reactions leading to DNA synthesis can catalyze the kinase reaction.

The aim of the experiments reported here was to investigate whether the nucleoside diphosphate kinase reaction was catalyzed by the ribonucleotide reductase proteins or by a different enzyme and to determine if the association between ribonucleotide reductase and the kinase could have a physiological meaning.

MATERIALS AND METHODS

Sources for common nucleotides (8) and nucleotide analogues (6) as well as the purification of them (6) has been described. The purity of all nucleotides was at least 97% as determined by paper (9, 10) or thin layer (11) chromatography. Chromatographic materials were obtained from the same sources as earlier (5).

Copyright © 1976 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Enzyme assays - Ribonucleotide reductase activity was measured with an NADPH oxidation method (5). One unit is that amount of enzyme which catalyzes the reduction of 1 nmole of CDP per min. under the conditions of the assay.

Nucleoside diphosphate kinase activity was usually measured by incubating 2.5 nmoles of (^{3}H)ADP (specific activity $2x10^5$ cpm per nmole), 50 nmoles of ATP, 2.5 μmoles of Tris buffer pH 7.6, 0.5 μmole of $MgCl_2$, 0.1 μmole of β-mercaptoethanol and protein fraction for 30 min. at 37°C in a final volume of 0.05 ml. Reactions were stopped by addition of 5 μl of 4 M perchloric acid. The samples were centrifuged and the supernatants were neutralized with 4 M KOH. After removal of the precipitated perchlorate by centrifugation, 50 nmoles each of adenosine mono-, di- and triphosphate were added as markers and the mixtures were chromatographed on Whatman 3 MM paper in an isobutyric acid medium (9). Nucleotides were localized under UV light and pieces of equal size were cut from the dried papers and analyzed for radioactivity in 0.5 ml of Soluene scintillation fluid (Packard). One unit is that amount of nucleoside diphosphate kinase which catalyzes the formation of 1 nmole of (^{3}H)ATP per min. under these conditions.

RESULTS

Nucleoside Diphosphate Kinase Activity in Preparations of Proteins B1 and B2

Highly purified preparations of the ribonucleotide reductase subunits (B1 and B2) contain an activity that converts ribonucleoside diphosphates to the corresponding triphosphates. All common nucleoside triphosphates tested could act as phosphate donors, but a diphosphate was inactive when the reaction was catalyzed by either a preparation of protein B2 (Table I) or B1 (not shown). The reaction had an absolute requirement for Mg^{2+} ions and ADP, GDP and CDP were all converted to the corresponding ribonucleoside triphosphates. No formation of ribonucleoside monophosphate occurred.

Nucleoside triphosphate analogues containing either a methylene bridge between the β- and γ-phosphates (12) or having one oxygen at the γ-phosphate replaced by a sulfur (13) were tested in the reaction. None of the analogues stimulated the formation of (^{3}H)GTP from (^{3}H)GDP (Table II), indicating that the reaction involves a transfer of the γ-phosphate group to the diphosphate. The results of the above experiments are consistent with the reaction catalyzed by a nucleoside diphosphate kinase (14): $NDP + N'TP \rightleftarrows NTP + N'DP$.

Nucleoside Diphosphate Kinase Activity during Purification of Ribonucleotide Reductase

The distribution of the nucleoside diphosphate kinase and ribonucleotide reductase activities was determined in the different chromatographic steps in the purification of ribonucleotide reductase to determine if the activities copurified. Ribonucleotide reductase was purified as described by Brown et al. (5) with the modification of Thelander (4).

Table I Substrate requirements of the kinase activity present in highly purified preparations of protein B2.

	A		B	
Phosphate donor	%(^{3}H)ATP formed	%(^{3}H)AMP formed	%(^{3}H)ATP formed	%(^{3}H)AMP formed
ATP	21.2	1.1	39.2	1.4
dATP	24.3	1.3	45.5	1.4
dTTP	11.7	1.6	21.2	1.6
dGTP	6.8	1.4	13.9	1.7
dCTP	3.9	1.6	7.8	1.7
dTDP	0.6	1.7	0.8	1.7
none	0.8	1.9	0.8	1.9

Concentrations of nucleoside triphosphates or dTDP were 1.0×10^{-3} M and of (^{3}H)ADP 5.2×10^{-5} M. Two concentrations of protein B2 from Sephadex G-200 gel filtration were tested: A 0.13 mg/ml and B 0.26 mg/ml and incubations were performed according to Methods.

Table II. Nucleoside triphosphate analogues as phosphate donors in the kinase reaction.

Experiment number	Nucleoside triphosphate	Concentration of nucleoside triphosphate M$\times 10^4$	%(^{3}H)GTP formed
I	dATP	0.5 - 20	4.5 - 33
	dAMP-P(CH_2)P[1]	0.5 - 20	0.0 - 0.1
II	dTTP	0.007 - 7.3	0.0 - 64
	dTMP-P(CH_2)P[1]	0.007 - 7.3	0.0 - 2.4
III	dGTP	0.01 - 10	0.6 - 65
	dGMP-P(CH)$_2$)P[1]	0.01 - 10	0.0 - 1.5
IV	dGTP	0.01 - 10	1.5 - 33
	dGTPγS[2]	0.01 - 10	0.0 - 2.0
V	ATP	0.1 - 100	52 - 80
	AMP-P(CH_2)P[1]	0.1 - 100	0.8 - 3.0

[1] Nucleoside triphosphate with a methylene bridge between the β- and γ-phosphates.

[2] Nucleoside triphosphate with one oxygen at the γ-phosphate replaced by a sulfur.

Incubations were performed for 45 min. at 0°C and contained: 0.05 M Tris buffer pH 7.6, 0.01 M $MgCl_2$, 1.1×10^{-5} M (^{3}H)GDP (specific activity = 10^5 cpm/nmole), 3.5 mg/ml of protein B1 from one of the last two purification steps and nucleoside triphosphates or analogues of those indicated above at four to five different concentrations. Reactions were treated as described under Methods.

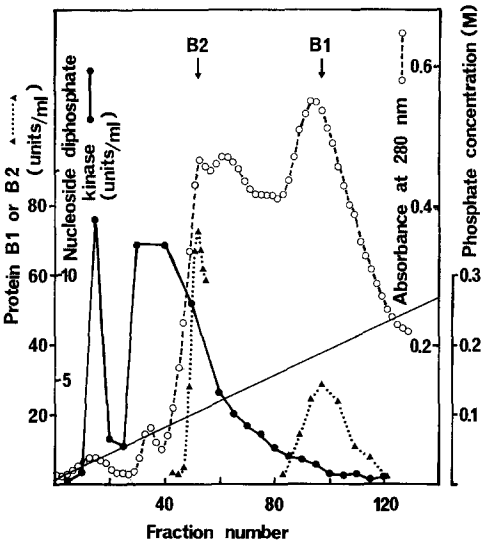

Fig. 1. Hydroxylapatite chromatography of ribonucleotide reductase. Protein (1.3 g) from first DEAE-cellulose was applied to a 6 x 25 cm hydroxylapatite column and eluted with a linear gradient of 0.01-0.4 M potassium phosphate buffer, pH 6.4, containing 1 mM dithiothreitol. Fractions of 30 ml were collected and analyzed for ribonucleotide reductase and nucleoside diphosphate kinase activities as described under Methods.

The first purification step studied was DEAE-cellulose chromatography at pH 7.0. Nucleoside diphosphate kinase eluted as a broad peak covering the region where ribonucleotide reductase eluted. The pooled ribonucleotide reductase fractions contained about 20% of the kinase activity.

After DEAE-cellulose chromatography ribonucleotide reductase was applied to a hydroxylapatite column which separates the B1 and B2 proteins from each other. As seen in Fig. 1 a peak of nucleoside diphosphate kinase activity eluted before and trailed out into the fractions containing the ribonucleotide reductase proteins. Protein B2 contained more kinase activity than B1 since it eluted at a lower ionic strength. About 10% of the nucleoside diphosphate kinase applied was recovered with the ribonucleotide reductase proteins (Table III). After hydroxylapatite chromatography proteins B1 and B2 were purified separately.

Correlation between Protein B1 and Nucleoside Diphosphate Kinase Activity

Protein B1 was further purified by affinity chromatography on a dATP-Sepharose column (4). About 2% of the kinase activity applied to the column was eluted together with protein B1, while the rest was removed during the washing procedure (Table III). The final step in the purification

Table III. Recovery of nucleoside diphosphate kinase in the purification steps of the ribonucleotide reductase proteins.

Purification step	mg protein	Units ribonucl. reductase	Yield of ribonucl. reductase %	Units kinase	Yield of kinase %	Units of kinase per mg protein
Protein B1: Hydroxyl-apatite	244	25000	25	490	3.5	2.01
dATP Sepharose	46	23000	23	10.0	0.071	0.22
Sephadex G-200	23	13100	13	4.67	0.033	0.20
Protein B2: Hydroxyl-apatite	38	5050	42	860	6.2	22.6
DEAE-cellulose	9.5	3700	31	21.0	0.15	2.2
Sephadex G-200	6.1	1990	17	10.2	0.074	1.7

A total of 835 mg of ribonucleotide reductase from the first DEAE-cellulose column was used as starting material. The yields are based on the amount of each activity applied to the hydroxylapatite column. Assays described under Methods were used. An absorbance of 1.0 at 280 mm (1 cm light path) was assumed to correspond to 1 mg/ml for protein B1 and 0.7 mg/ml for protein B2 (5).

of B1 was chromatography on Sephadex G-200 in a buffer containing 0.2 M NaCl. The two enzymatic activities eluted together from this column and the amount of nucleoside diphosphate kinase activity per mg of protein B1 remained unchanged (Table III).

A preparation of protein B1 from Sephadex G-200 was subjected to re-chromatography on hydroxylapatite under the same conditions as before where the ribonucleotide reductase and kinase activities were eluted at different ionic strengths (Fig. 1). The amount of nucleoside diphosphate kinase activity in the B1 preparation remained unchanged after this treatment. I conclude that either the two reactions are catalyzed by the same molecule or there is a tight binding between protein B1 and a different protein catalyzing the kinase reaction.

Correlation between Protein B2 and Nucleoside Diphosphate Kinase Activity

Protein B2 from the hydroxylapatite chromatography was applied to a

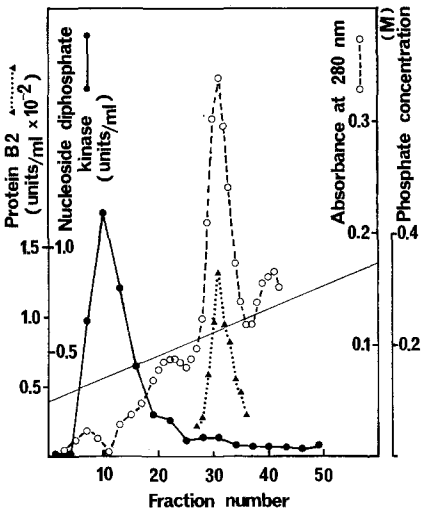

Fig. 2. Chromatography of protein B2 on a second DEAE-cellulose column. Protein (75 mg) from hydroxylapatite step was put on a 3x7 cm DEAE-cellulose column and eluted with a linear gradient of 0.1-0.35 M potassium phosphate pH 6.4. Fractions of 20 ml were collected and assayed for the two enzymatic activities. For measurements of nucleoside diphosphate kinase activity, the incubation mixtures contained 1.0×10^{-5} M (^{3}H)CDP (specific activity 2×10^5 cpm per nmole), 1 mM ATP, 0.01 M $MgCl_2$, 0.05 M Tris buffer pH 7.6 and 5 µl of protein fraction. The final volume was 10 µl and incubations were carried out for 15 min. at 37°. Reactions were stopped by precipitation with perchloric acid and analyzed as described in Methods. One unit is that amount of enzyme which phosphorylates 1 nmole of (^{3}H)CDP per min.

second DEAE-cellulose column and eluted with a linear gradient of potassium phosphate buffer at pH 6.4. The majority of the nucleoside diphosphate kinase activity eluted before protein B2 (Fig. 2) and the pooled fractions containing B2 (21-35) contained approximately 2% of the kinase activity applied to the column (Table III). The last purification step for B2 Sephadex G-200 chromatography in 0.05 M Tris buffer pH 7.6 containing 0.2 M NaCl, gave little further separation between the two activities (Table III). When a protein B2 preparation from Sephadex G-200 was rechromatographed on DEAE-cellulose at pH 6.4, no further separation of B2 and the nucleoside diphosphate kinase activity was obtained.

In a further attempt to separate protein B2 from the kinase activity, a protein B2 preparation from the second (pH 6.4) DEAE-cellulose step was subjected to preparative polyacrylamide gel electrophoresis (5). The result (shown in Fig. 3) was that the two enzymatic activities migrated with

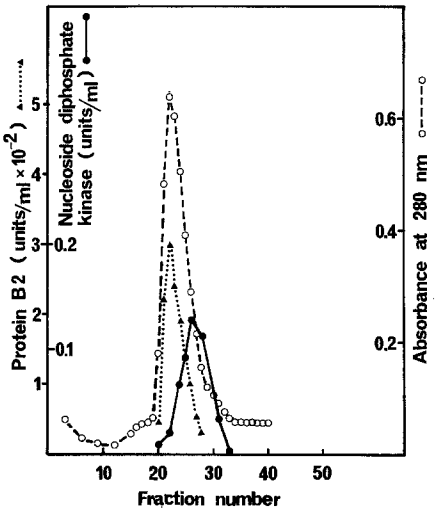

Fig. 3. Preparative gel electrophoresis of protein B2. Protein (18.4 mg) from second DEAE-cellulose was subjected to gel electrophoresis under the same conditions as described before (5). Fractions of 5 ml were collected and analyzed for the two enzymatic activities. The determination of nucleoside diphosphate kinase was carried out as described in Fig. 2.

different mobilities, meaning that the nucleoside diphosphate kinase and protein B2 activities are associated with different molecules.

DISCUSSION

The association of nucleoside diphosphate kinase activity with ribonucleotide reductase would seem to be advantageous for the cell, since the deoxyribonucleoside diphosphates produced by ribonucleotide reductase have to be phosphorylated to serve as substrates for DNA polymerase. Measurement of nucleoside diphosphate kinase activity in chromatograms of the ribonucleotide reductase purification showed that the major part of the kinase was separated from the ribonucleotide reductase subunits (proteins B1 and B2) (Table III). Sephadex G-200 gel filtration or rechromatography on hydroxylapatite or DEAE-cellulose, however, did not remove the remaining kinase activity, suggesting that either (1) the nucleoside diphosphate kinase reaction is catalyzed by both a separate enzyme and also by proteins B1 and B2, or (2) that there is a strong binding between the ribonucleotide reductase proteins and a kinase enzyme. It was, however, possible to separate the kinase activity from protein B2 by gel electrophoresis (Fig. 3) and a preparation of protein B1 from a λ lysogenic Escherichia coli strain producing large amounts of ribonucleo-

tide reductase[1] contained half as much kinase activity as the preparations above (not shown). It is therefore probable that the nucleoside diphosphate kinase reaction is not catalyzed by the ribonucleotide reductase subunits per se, but by a separate enzyme that binds tightly to proteins B1 and B2.

The tight binding between the two enzymes might indicate that they are physically close in the cell. It has been reported that deoxyribonucleoside diphosphates are incorporated into DNA in a bacterial in vitro system, probably as a result of in situ phosphorylation to triphosphates (15, 16). These reports are in agreement with the finding by Miller and Wells (7) of nucleoside diphosphate kinase activities in highly purified preparations of DNA polymerases. It is known that ribonucleotide reductase is present in a highly active form in the cell that is lost upon purification, indicating that a stabilizing structure is destroyed when the enzyme is isolated (17). On the basis of studies with the temperature sensitive DNA replication mutant dnaF which has a defective ribonucleotide reductase, Dermody and coworkers (18) suggest that ribonucleotide reductase is part of the replication complex and has a function in funneling nucleotides to DNA polymerase III.

ACKNOWLEDGEMENTS

This study was supported by grants from the Medical Faculty of the Karolinska Institute and the Swedish Medical Research Council to Peter Reichard.

REFERENCES

1. Fuchs, J.A., Karlström, H.O., Warner, H.R. and Reichard, P. (1972) Nature New Biology 238, 69-71.
2. Larsson, A. and Neilands, J.B. (1966) Biochem. Biophys. Res. Commun. 25, 222-226.
3. Reichard, P. (1967) in Biosynthesis of Deoxyribose, Ciba Lectures in Biochemistry, John Wiley and Sons Inc., New York.
4. Thelander, L. (1974) J. Biol. Chem. 249, 4858-4862.
5. Brown, N.C., Canellakis, Z.N., Lundin, B., Reichard, P. and Thelander, L. (1969) Eur. J. Biochem. 9, 561-573.
6. von Döbeln, U. and Reichard, P. (1976) J. Biol. Chem. 251, 3616-3622.
7. Miller, L.K. and Wells, R.O. (1971) Proc. Natl. Acad. Sci., U.S. 68, 2298-2302.
8. Brown, N.C. and Reichard, P. (1969) J. Mol. Biol. 46, 39-55.
9. Krebs, H.A. and Hems, R. (1953) Biochim. Biophys. Acta 12, 172-180.
10. Reichard, P. (1958) Acta Chem. Scand. 12, 2048-2049.

[1] Eriksson, S., Sjöberg, B.-M., Hahne, S. and Karlström, O., to be published.

11. von Döbeln, U. and Eckstein, F. (1974) Eur. J. Biochem. 43, 215-220.
12. Englund, P. T., Hubermann, J. A., Jovin, T. M. and Kornberg, A. (1969) J. Biol. Chem. 244, 3038-3044.
13. Goody, R. S. and Eckstein, F. (1971) J. Amer. Chem. Soc. 93, 6252-6257.
14. Edlund, B. (1971) Acta Chem. Scand. 25, 1370-1376.
15. Schaller, H., Otto, B., Nüsslein, V., Huf, J., Hermann, R., and Bonhoeffer, F. (1972) J. Mol. Biol. 63, 183-200.
16. Pollock, J. M. and Werner, R. (1975) Biochem. Biophys. Res. Commun. 63, 699-705.
17. Eriksson, S. (1975) Eur. J. Biochem. 56, 289-294.
18. Dermody, J. J., Bourguignon, G. J., Foglesong, P. D. and Sternglanz, R. (1974) Biochem. Biophys. Res. Commun. 61, 1340-1347.