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STUDIES ON A MEMBRANE-BOUND AND SOLUBILIZED RIBONUCLEOTIDE REDUCTASE PREPARATION FROM *ESCHERICHIA COLI* TAU$^-$

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Summary

Ribonucleotide reductase has been shown to be associated with the DNA-membrane complex in *Escherichia coli* TAU$^-$ cells. The membrane-bound enzyme has been released in a soluble form using a combined treatment of 1% sarcosyl (pH 8.0) and 1% sodium deoxycholate (pH 6.5). Allotropic differences in the modulatory effects of ATP, Mg^{2+}, EDTA and dithiothreitol on the membrane-bound and solubilized enzyme activity are discussed.

Introduction

The role of bacterial cell membrane in DNA replication and cell division has been reported [1—3]. Ribonucleotide reductase (2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1), an enzyme uniquely responsible for the reduction of ribonucleotides to deoxyribonucleotides (thus providing necessary precursors for DNA synthesis) is a product of DNA F gene [4] and maps close to the DNA A, B, C, D, and E genes involved in DNA synthesis on the *Escherichia coli* chromosome. The increase in the levels of this enzyme parallels DNA synthesis in serum stimulated mouse embryo cells [5], during the S phase in L cells [6] and during cleavage in sea urchin embryogenesis [7]. Data presented in this paper provide evidence for the presence of this enzyme attached to the DNA-membrane complex of *E. coli* TAU$^-$ cells. A comparative study of the properties of the membrane-bound enzyme and its solubilized counterpart has also been presented.

Methods

E. coli TAU$^-$ cells were grown in M9 medium [8] supplemented with 0.5% casamino acids, 4 mg/ml thymine, 5 mg/ml adenine sulfate and 10 mg/ml uracil. A 10% innoculum (overnight culture) was used to initiate the culture which was grown to a cell count of $5 \cdot 10^8$ cells/ml (2.5 h at 37°C with aeration) after which the cells were collected by centrifugation (10 000 × *g*), washed with sterile saline and incubated for 1 h in fresh medium containing no thymine [9].

Membrane preparation. The cells were collected and the membrane complex was prepared according to the method of Majumdar et al. [10] except that incubation with EDTA and lysozyme was for 5 min instead of 15 min. The method provides cell membrane preparation with DNA complexed to RNA, protein and lipids. Gross composition of the DNA-membrane complex preparation isolated from exponentially grown *E. coli* TAU$^-$ cells has been reported earlier from our laboratory [11]; 85% of the total cellular DNA was found to be associated with the membrane complex together with 56% RNA, 38% protein and 55% lipids.

Solubilization of enzyme. Attempts were made at solubilizing the ribonucleotide reductase activity of the DNA-membrane preparation with various ionic and nonionic detergents. Treatments with 1% sarcosyl, 1% sodium deoxycholate, 1% Triton X-100 and 1% Brij 58 were tried under varying conditions and combinations. The DNA-membrane complex corresponding to $2.5 \cdot 10^8$ cells was treated directly with the extracting detergents by stirring in ice for 2—5 min followed by centrifugation at 15 000 × *g* for 10 min. During solubilization 10 mM $MgCl_2$ was omitted in the buffer, since its presence rendered the process more difficult.

Enzyme assay. The reaction mixture, in a total volume of 150 µl, contained 12 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM ATP, 0.5 mM [^{3}H]CDP ($1 \cdot 10^6$ cpm), 15 mM Tris-HCl buffer (pH 7.8) and 0.2—0.5 mg enzyme protein. After a 20 min incubation period the reaction was terminated by addition of 1 M cold $HClO_4$. The product dCDP was separated from the unreacted CDP on Dowex 50 (H^+) columns after hydrolysis (to the corresponding nucleoside monophosphates) as described by Reichard et al. [12]. 0.5-ml aliquots, representing the reduced product formed, were neutralized and counted for radioactivity using Bray's solution [13]. Results were calculated for the 20 min reaction.

Results

Ribonucleotide reductase has been well studied in bacterial and mammalian systems. Since the enzyme levels are very low in these cells, studies have been done in thymine-starved cells [9] where it is induced several fold higher. Of the various detergents tried for solubilization of ribonucleotide reductase from the DNA-membrane complex, 1% sarcosyl and 1% sodium deoxycholate partially released the enzyme activity. Recoveries were poor with Brij 58 and Triton X-100. A combined treatment of the membrane fraction with sarcosyl and sodium deoxycholate under various pH conditions was next attempted and the

TABLE I

EFFECT OF DETERGENTS AND pH ON THE SOLUBILIZATION OF *E. coli* RIBONUCLEOTIDE REDUCTASE ACTIVITY

The membrane preparation was treated with 1 ml extracting medium stirred in ice for 2—5 min and centrifuged for 10 min at 15 000 × *g*. 100 μl of the extract were used for the (20 min) assay.

Starting preparation	Extraction treatment	Enzyme preparation	Enzyme activity (nmol CDP reduced per mg protein)
Whole membrane	—	Whole membrane in Tris-HCl buffer (pH 7.8)	2.420
Whole membrane	1% sarcosyl at pH 6.5	1% sarcosyl extract (pH 6.5)	0.075
1% sarcosyl (pH 6.5) treated pellet	1% sodium deoxycholate at pH 6.5	1% sodium deoxycholate extract (pH 6.5)	0.235
1% sarcosyl (pH 6.5) treated pellet	1% sodium deoxycholate at pH 8.0	1% sodium deoxycholate extract (pH 8.0)	2.150
Whole membrane	1% sarcosyl at pH 8.0	1% sarcosyl extract (pH 8.0)	1.020
1% sarcosyl (pH 8.0) treated pellet	1% sodium deoxycholate at pH 6.5	1% sodium deoxycholate extract (pH 6.5)	3.670
1% sarcosyl (pH 8.0) treated pellet	1% sodium deoxycholate at pH 8.0	1% sodium deoxycholate extract (pH 8.0)	—

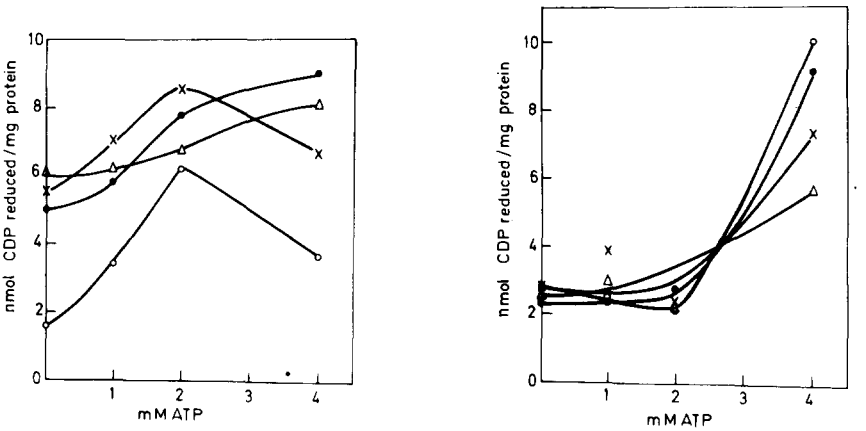

Fig. 1. Effect of ATP on *E. coli* membrane bound ribonucleotide reductase activity in presence of 0 (○———○), 10 (●———●), 15 (X———X) and 20 mM (△———△) $MgCl_2$.

Fig. 2. Effect of ATP on the *E. coli* solubilized ribonucleotide reductase activity in presence of 0 (○———○), 10 (●———●), 15 (X———X) and 20 mM (△———△) $MgCl_2$.

results are shown in Table I. Treatment with 1% sarcosyl at pH 8.0 followed by extraction with sodium deoxycholate at pH 6.5 was found to be the best, resulting in solubilization of 60% of the whole membrane activity. This method was therefore used for obtaining the solubilized enzyme routinely.

Sigmoid curves were obtained when activities of the membrane bound and the soluble enzyme were plotted against increasing substrate concentration indicating the allosteric nature of the enzyme. Both the preparations were maximally active at around 0.5 mM substrate concentration.

The membrane-bound enzyme is active in the absence of extraneous Mg^{2+}, optimal activity being reached in the presence of 2 mM ATP (Fig. 1). Mg^{2+}

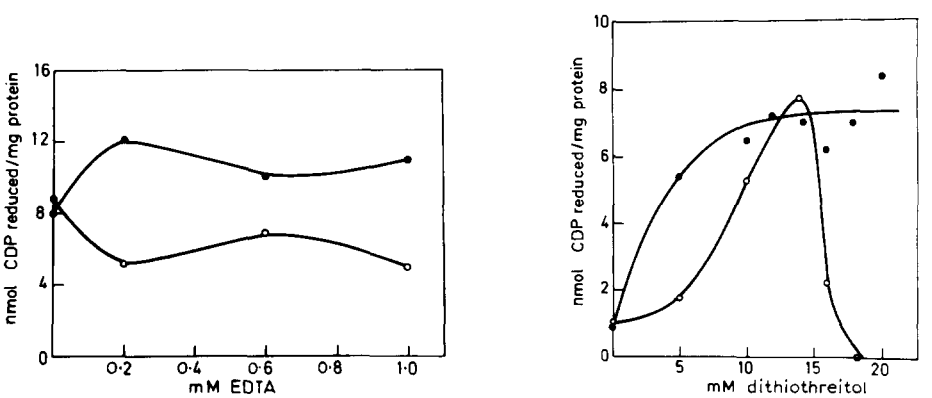

Fig. 3. Effect of EDTA on *E. coli* ribonucleotide reductase activity of membrane-bound (●———●) and solubilized (○———○) enzyme preparations.

Fig. 4. Effect of dithiothreitol on the *E. coli* ribonucleotide reductase activity of membrane-bound (●———●) and solubilized (○———○) enzyme preparations.

(10—20 mM) also promoted activity in the absence of ATP. In their combined presence the stimulatory effects are additive. Optimal activity was obtained in the presence of 10—15 mM Mg^{2+} and 2 mM ATP. With the soluble enzyme the pattern (Fig. 2) of stimulation was distinctly different. Addition of up to 20 mM Mg^{2+} in the presence of up to 2 mM ATP have no effect. Higher concentrations of ATP (around 4 mM) alone, stimulated the enzyme 4—5-fold; however, in the added presence of Mg^{2+} the stimulation by 4 mM ATP is less evident, being only 2-fold in the presence of 20 mM Mg^{2+}.

The membrane-bound enzyme was stimulated (50%) by 0.2 mM EDTA while the solubilized enzyme was inhibited to a similar extent by the same EDTA concentration (Fig. 3). Both the membrane-bound and the solubilized enzymes are optimally stimulated by approx. 13—15 mM dithiothreitol. While the activity of the membrane-bound enzyme increases with increasing dithiothreitol concentration reaching a plateau above 12 mM, the activity of the soluble enzyme falls sharply below or above the optimal dithiothreitol concentration (Fig. 4).

Discussion

In this report we have shown that the enzyme ribonucleotide reductase activity in *E. coli* TAU^- cells is associated with the DNA-membrane complex. It was possible to maximally solubilize the enzyme by extracting the 1% sarcosyl (pH 8.0) pretreated membrane preparation with 1% sodium deoxycholate at pH 6.5. Elford [14] had earlier reported the association of ribonucleotide reductase with the membrane fragment of the post microsomal supernatant of regenerating rat liver and Novikoff hepatoma cells. Deoxyribonucleotide kinases and the DNA polymerase have been detected in the DNA-membrane complex obtained from *Pneumococci* [15]. A protein-nucleic acid complex having most of the enzymes essential for discontinuous DNA replication as well as RNA-linked Okazaki fragments has been isolated from *Tetrahymena pyriformis* [16]. Similar complexes have also been described in bacteriophage T4-infected cells by Reddy and Mathews [17]. These findings tend to support the suggestion [18] that ribonucleotide reductase as well as other enzyme involved in nucleotide interconversions and DNA synthesis could be held close together bound to the membrane as a coordinated replicating complex.

Ribonucleotide reductase from *E. coli* is an allosteric enzyme consisting of two nonidentical, separately inactive subunits, called B1 and B2, each consisting of two identical or very similar polypeptide chains. The two subunits in the presence of Mg^{2+} form a complex representing the active enzyme [19]. Protein B1 contributes with its active SH-groups and provides binding sites for the ribonucleoside diphosphate substrates [20] and for the nucleoside triphosphate effectors [21]. Protein B2 contains the bound non-heme iron and an organic free radical essential for activity [19,22].

The striking difference in the requirements of Mg^{2+} for optimal activity of the membrane-bound and the solubilized enzyme is evident from a comparative study of their behaviour (Figs. 1 and 2). It would seem that the extraction procedure we have used for solubilization of the enzyme does not disaggregate the Mg^{2+}-held multisubunit structure of the active enzyme, since extraneous

Mg^{2+} is not required for activity. The enzyme is more dependent on the binding of the positive effector ATP for activity. On the other hand the membrane-bound enzyme is amenable to the modulating effects of both the subunit aggregating property of Mg^{2+} and the positive effector ATP. The bell-shaped response of the activity of the solubilized enzyme to increasing dithiothreitol concentration is also different from the response of the membrane-bound enzyme. Similarly, EDTA addition enhances the membrane-bound activity while inhibiting the solubilized enzyme. Such differences in the properties of an enzyme when it is moved from its membrane location into an aqueous solution are known and were termed 'allotropic' properties by Racker [23]. A membrane-bound ATPase from *E. coli* which behaves differently when released in a soluble form has also been characterized [24].

Ribonucleotide reductase obtained by mechanical disintegration of cells is reported to be less active than that prepared by the cellophane membrane disc method of Eriksson [25], who had indicated that the intracellular form of the enzyme consisted of a tight complex of proteins B1 and B2, possibly stabilized by other intracellular structures. The dissociation of this complex in vitro could cause secondary changes of the protein, resulting in a less active enzyme upon reassociation. Solubilization with ionic detergents, as described here, does not destroy the structural features essential for activity of the solubilized enzyme. While conventional purification procedures have greatly aided in characterizing the enzyme, its behaviour within the constraints of its native membrane-bound environment may be more relevant to understanding its in situ functioning.

References

1 Ganesan, A.T. and Lederberg, J. (1965) Biochim. Biophys. Res. Commun. 18, 824—835
2 Smith, D.W. and Hanawalt, P.C. (1967) Biochim. Biophys. Acta 149, 519—531
3 Ryter, A., Hirota, Y. and Jacob, F. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 669—676
4 Fuchs, J.A., Karlstrom, H.O., Warner, H.R. and Reichard, P. (1972) Nat. N. Biol. 238, 69—71
5 Nordenskjold, B.A., Skoog, L., Brown, N.C. and Reichard, P. (1970) J. Biol. Chem. 245, 5360—5368
6 Turner, M.K., Abrams, R. and Lieberman, I. (1968) J. Biol. Chem. 243, 3725—3728
7 Noronha, J.M., Sheys, G.H. and Buchanan, J.M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2006—2010
8 Lark, K.G., Repko, T. and Hoffman, E.J. (1963) Biochim. Biophys. Acta 76, 9—24
9 Biswas, C., Hardy, J. and Beck, W.S. (1965) J. Biol. Chem. 240, 3631—3640
10 Majumdar, C. and Frankel, F.R. (1973) Nature 243, 33—36
11 Nair, C.K.K., Pradhan, D.S. and Sreenivasan, A. (1975) Proceedings of Symposium on Structural and Functional aspects of Chromosomes, March 25—26, 1975, Bombay, ('Basic Sciences-Biology and Medical-Committee' of the Department of Atomic Energy, Government of India, pp. 162—174
12 Reichard, P., Baldesten, A. and Rutberg, L. (1961) J. Biol. Chem. 236, 1150—1157
13 Brays, G.A. (1960) Anal. Biochem. 1, 279—285
14 Elford, H.L. (1974) Arch. Biochem. Biophys. 163, 537—543
15 Firshein, W. and Gillmor, R.G. (1970) Science 169, 66—68
16 Westergaard, O. and Johnson, B. (1973) Biochem. Biophys. Res. Commun. 55, 341—349
17 Reddy, G.P.V. and Mathews, C.K. (1978) J. Biol. Chem. 253, 3461—3467
18 Baril, E., Baril, B. and Elford, H. (1972) Proc. Am. Ass. Cancer Res. 13, 84
19 Thelander, L. (1973) J. Biol. Chem. 248, 4591—4601
20 Dobeln, U.V. and Reichard, P. (1976) J. Biol. Chem. 251, 3616—3622
21 Brown, N.C. and Reichard, P. (1969) J. Mol. Biol. 46, 39—55
22 Atkin, C.L., Thelander, L., Reichard, P. and Lang, G. (1973) J. Biol. Chem. 248, 7464—7472
23 Racker, E. (1967) Fed. Proc. 26, 1335—1340
24 Roisin, M.P. and Kepes, A. (1973) Biochim. Biophys. Acta 305, 249—259
25 Eriksson, S. (1975) Eur. J. Biochem. 56, 289—294