

FLAVODOXIN IS REQUIRED FOR THE ACTIVATION OF THE ANAEROBIC RIBONUCLEOTIDE REDUCTASE

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Summary. The inactive anaerobic ribonucleotide reductase from *Escherichia coli* is transformed by a multienzyme system and S-adenosylmethionine + NADPH into a radical protein that is enzymatically active. One of the activating enzyme components was earlier shown to be ferredoxin (flavodoxin):NADP⁺ reductase. Here we present evidence that flavodoxin, but not ferredoxin, also is a component of the system. Light reduced deazaflavin can substitute for the flavodoxin system. An additional unidentified low-molecular weight component further stimulates the reaction. © 1993 Academic Press, Inc.

During anaerobic growth of *Escherichia coli* the formation of the deoxyribonucleotides required for DNA synthesis is catalyzed by a special anaerobically induced ribonucleoside triphosphate reductase, different from the well characterized aerobic diphosphate reductase (1). As isolated, the anaerobic enzyme is inactive and requires activation resulting in the formation of a protein radical, probably located on a glycine residue present in the C-terminal region of the enzyme (2). Activation occurs with S-adenosylmethionine (AdoMet) and NADPH by a multienzyme system. One component was recently identified as ferredoxin (flavodoxin) reductase (3). Other components are present in a Chelex-treated boiled extract of *E. coli*, provisionally named RT (4).

In this communication we report that one of the active components present in RT is flavodoxin. In addition, RT contains one or several low molecular weight components that have the ability to stimulate the activity

of flavodoxin. The extent of stimulation varies greatly with different preparations of the anaerobic reductase.

MATERIALS AND METHODS

Materials. Anaerobic ribonucleotide reductase (4), methionine synthase (5), ferredoxin (flavodoxin) reductase (3) and fraction RT (4) were prepared as described. Ferredoxin was a gift from Dr. L.E. Vickery, Dept. of Physiology and Biophysics, University of California. Deazaflavin was a gift from Dr. A. Ehrenberg, Dept. of Biophysics, University of Stockholm.

Flavodoxin was purified from an extract of the *E. coli* strain DH01 described below, after IPTG induction of the bacteria. Purification involved a combination of chromatography on DEAE Fast Flow Sepharose (Sigma) and MonoQ HR 16/10 (Pharmacia). Details of the procedure can be obtained on request. Homogenous flavodoxin with an A274/A467 ratio of 5.8 was obtained.

Construction of an overexpression vector for flavodoxin. The flavodoxin coding sequence was amplified by PCR and introduced into pTRC99A (Pharmacia). The following primers were used for amplification of template DNA containing the *fldA* gene (pRMEcoRVIII) (5): 5'-CGGGATCCATGGCTATCACTGGGCATCTTT-3' and 5'-CGGAATTCTAGATCATCAGGCATTGAGAATTCGTC-3'. The underlined sequence in the first primer is the coding sequence for the first seven amino acids of flavodoxin, and *Nco* I and *Bam*HI sites have been placed upstream of this sequence. The underlined sequence in the second primer is complementary to the last six amino acids of flavodoxin and the stop codon. A second stop codon and *Eco*RI and *Xba* I sites have been incorporated upstream of this region. The 550 bp PCR product was cleaved with *Nco* I and *Eco*RI and ligated into pTRC99A vector that had been digested with the same restriction enzymes. The religated vector, pDH1, was introduced into strain DH5aF' by transformation, creating strain DH01.

Separation of RTF and RTR. A preparation of RT (0.5 ml) concentrated 3 fold by evaporation was chromatographed on a 12 ml FPLC column of Superdex 75 (Kabi Pharmacia, Stockholm, Sweden) equilibrated with 50 mM Tris-Cl, pH 8.0. RTR was recovered between 3.5 and 5.0 ml, RTF between 11 and 13 ml after the start of the run.

Activation of anaerobic reductase. Details of the conditions were described earlier (7). Briefly, a fixed amount of enzyme (between 1 and 2 μ g) is preincubated anaerobically during an activation period of 60 min with RT, 0.4 μ g flavodoxin reductase, 1 mM NADPH, 0.5 μ M Adomet, 5 mM dithiothreitol and 30 mM KCl. In an anaerobic second step, 1.5 mM radioactive CTP, 2 mM ATP and 10 mM Mg ions are added and the amount of dCTP formed during a 20 min period is determined and serves as a measure of the activation that had occurred during the first step. When deazaflavin was used (5-20 μ M), a solution of the compound was first made anaerobic in the dark and subsequently added to

the anaerobic preincubation mixture. The reaction was started by irradiation with light and preincubation continued for 20 min.

Methionine synthase activity. Cobalamin-dependent methionine synthase (5) was assayed in 1 ml of 0.1 M potassium phosphate, pH 7.2, containing 38 μ M AdoMet, 25 mM dithiothreitol and 1 mM homocysteine. Enzymes were added in the following order: 50 nM ferredoxin (flavodoxin) reductase, 2.14 nM methionine synthase, 0-10 nM flavodoxin (or its equivalent in RT), and then 250 μ M (6-R,S)CH₃-H₄folate (1300 dpm/nmol). The samples were equilibrated with argon before adding methyltetrahydrofolate and then incubated 10 min at 37°C after addition of the methyltetrahydrofolate. The assay was terminated by boiling 2 min and then analyzed by passage of the solution over a 2 ml column of AGI x 8 Cl⁻ which retained methyltetrahydrofolate while methionine was eluted and counted (8).

RESULTS

RT can be separated into two components. Purification of RT on Superdex-75, a column that separates components according to size, resolved RT into two active fractions. One eluted at a position corresponding to a molecular mass of approximately 20 kDa (RTR), the other appeared together with the low-molecular weight material (RTF). A similar fractionation could be obtained by centrifugation in Centricon 10 tubes with RTR in the retentate and RTF in the filtrate. Alone, each of the two components gave some activation of the anaerobic reductase, but with most preparations of the enzyme maximal activity required the presence of both RTR and RTF.

The experiment shown in Table 1 demonstrates that the simultaneous addition of RTR and RTF gave a 6- or 10-fold higher activation than could be achieved with either RTR or RTF alone. The reaction also required the presence of ferredoxin (flavodoxin) reductase, NADPH and Adomet (data not shown). Flavodoxin substituted for RTR, but not for RTF (Table 1).

The anaerobic reductase was also activated by light-reduced deazaflavin. The compound substituted for the sum of RTR + ferredoxin (flavodoxin) reductase + NADPH (Table 2). No activity was found in the dark (not shown). The reaction with deazaflavin was also stimulated by RTF.

RTR is identical with flavodoxin. These results suggest that the flavodoxin system (flavodoxin + flavodoxin reductase + NADPH) functions during the activation of the anaerobic ribonucleotide reductase, possibly by reducing the Fe-S cluster of the enzyme (2,9). Light-reduced deazaflavin then can substitute for the flavodoxin system.

Table 1. Flavodoxin substitutes for one component of RT

Conditions	nmol dCTP/min	
	no fldx	with fldx
Complete	0.360	-
minus RTR	0.038	0.44
minus RTF	0.068	0.054

Activation of the ribonucleotide reductase was made under standard conditions, modified as indicated in the table, with or without 1 μ g flavodoxin (fldx).

To show that flavodoxin is identical with RTR we decided to analyze for the presence of flavodoxin in RT by its effect on the activity of methionine synthase. To this purpose identical preparations of flavodoxin and RT were analysed separately in Ann Arbor and Stockholm for their activity in the methionine synthase and anaerobic ribonucleotide reductase assay, respectively. The results depicted in Fig. 1 demonstrated that 2 μ l of RT corresponded to 0.2 μ g of flavodoxin in both the methionine synthase assay (Fig. 1A) and the ribonucleotide reductase assay (Fig. 1B). The anaerobic reductase used in the latter assay was only minimally stimulated by RTF. Ferredoxin showed no activity in either assay (data not shown).

When the preparation of flavodoxin used for the studies in Fig. 1A was boiled for 30 min it retained 30 % of its activity in the methionine synthase assay.

Table 2. Light-reduced deazaflavin can substitute for the flavodoxin system

Conditions	nmol dCTP/min	
	no daf	with daf
Complete	0.22	0.33
minus RTR	0.05	0.32
minus RTF	0.07	0.10
minus fldx reductase	0.01	0.27
minus NADPH	0.01	0.30

Incubations were under standard conditions except as noted in the table. Where indicated, light-reduced deazaflavin (daf) was present. fldx = flavodoxin.

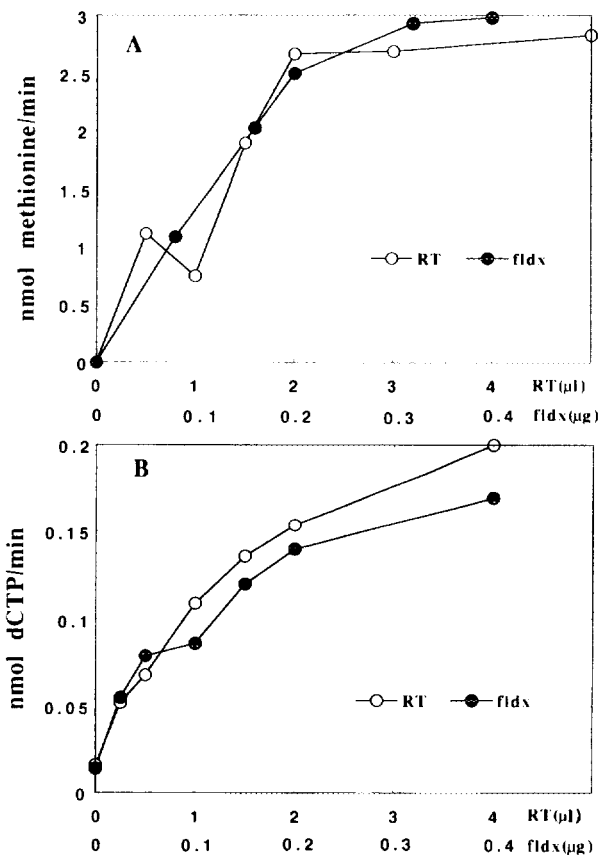


Fig. 1 - Comparison of RT and flavodoxin in methionine synthesis (A) and ribonucleotide reductase activation (B). Incubations were made under standard conditions for both enzyme reactions with increasing amounts of either RT (O) or flavodoxin (fldx = ●).

DISCUSSION

Our results demonstrate not only that flavodoxin can substitute for RTR, the protein component of RT, but also that flavodoxin is present in RT as determined by the methionine synthase assay. Furthermore, quantitation of flavodoxin by this assay closely matched the amount found with the ribonucleotide reductase activation assay. Taken together, these results provide strong evidence that RTR is identical with flavodoxin. Since we earlier showed that the activation of the anaerobic reductase besides RTR requires flavodoxin reductase and NADPH, our results now suggest that the whole flavodoxin system is involved in the process.

The flavodoxin system is required not only for methionine synthesis but also for the activation of the anaerobic pyruvate formate lyase (10). With the latter enzyme, light-activated deazaflavin substitutes for the flavodoxin system, as it does in our case, pointing to the similarity

between the activation of the anaerobic reductase and that of pyruvate formate lyase. In addition, the activity of both enzymes depends on the generation of a glycine radical (9,11).

Neither methionine synthase nor pyruvate formate lyase require a low molecular weight fraction similar to RTF. An understanding of the chemical nature of RTF and of the variability of its effect with different preparations of the reductase awaits results of present efforts aiming at its purification.

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REFERENCES

- 1 - Fontecave, M., Nordlund, P., Eklund, H. and Reichard, P. (1992) *Adv. in Enzymol. Relat. Areas Mol. Biol.* **65**, 147-183.
- 2 - Reichard, P. (1993) *J. Biol. Chem.* **268**, 8383-8386.
- 3 - Bianchi, V., Reichard, P., Eliasson, R., Pontis, E., Krook, M., Jörnvall, H. and Haggård-Ljungquist, E. (1993) *J. Bacteriol.* **175**, 1590-1595.
- 4 - Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jörnvall, H., Krook, M. and Reichard, P. (1992), *J. Biol. Chem.*, **267**, 25541-25547.
- 5 - Bannerjee, R.V., Johnston, N.L., Sobeski, J.K., Datta, P. and Matthews, R.G. (1989) *J. Biol. Chem.* **264**, 13888-13895.
- 6 - Osborne, C., Chen, L.M. and Matthews, R.G. (1991) *J. Bacteriol.* **173**, 1729-1737.
- 7 - Harder, J., Eliasson, R., Pontis, R., Ballinger, M.D. and Reichard, P. (1992) *J. Biol. Chem.* **267**, 25548-25552.
- 8 - Weissbach, H., Peterkovsky, A., Redfield, B.G. and Dickerman, H. (1963) *J. Biol. Chem.* **238**, 3318-3324.
- 9 - Mulliez, E., Fontecave, M., Gaillard, J. and Reichard, P. (1993) *J. Biol. Chem.* **268**, 2296-2299.
- 10 - Knappe, J. and Sawers, G. (1990) *FEMS Microbiol. Rev.* **75**, 383-398.
- 11 - Wagner, A.F.V., Frey, M., Neugebauer, F.A., Schäfer, W. and Knappe, J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 996-1000.