THIOREDOXIN FROM LACTOBACILLUS LEICHMANNII AND ITS ROLE AS HYDROGEN DONOR FOR RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE

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In previous studies from this laboratory on the hydrogen donor specificity of the cobamide coenzyme-dependent ribonucleoside triphosphate reductase of Lactobacillus leichmannii it was demonstrated that various dithiols, capable of intramolecular cyclization on oxidation, can serve as hydrogen donors for the reaction (Vitols and Blakley, 1965). Although dihydrolipoate proved to be more effective than the other low molecular weight dithiols tested, the thioredoxin system of Escherichia coli was found to be even more efficient. Thioredoxin of E. coli (Laurent et al., 1964) is a small protein (mol. wt. 12,000) containing a disulfide bond which is reduced by NADPH in the presence of a specific enzyme, thioredoxin reductase (Moore et al., 1964). Reduced thioredoxin acts as the immediate hydrogen donor for the ribonucleoside triphosphate reductase as shown in reactions (1) and (2) :

$$\text{NADPH} + \text{Thioredoxin-S}_2 \xrightarrow[\text{reductase}]{\text{Thioredoxin}} \text{NADP} + \text{Thioredoxin-(SH)}_2 \quad (1)$$

$$\text{Thioredoxin-(SH)}_2 + \text{GTP} \xrightarrow[\text{Coenzyme-B}_{12}]{\text{Ribonucleotide reductase}} \text{Thioredoxin-S}_2 + \text{dGTP} \quad (2)$$

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These results have been recently confirmed by Beck and his associates (1966). The present communication describes the isolation from L. leichmannii of two protein fractions which have the properties of thioredoxin and thioredoxin reductase and which are fully active in reactions (1) and (2). These proteins probably constitute the physiological hydrogen donor system for ribonucleoside triphosphate reduction in this organism.

The thioredoxin system of E. coli is able to couple the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to the oxidation of NADPH (Laurent et al., 1964), and this reaction can be used to assay thioredoxin activity in bacterial extracts. The detection of such activity in crude extracts of L. leichmannii was greatly hampered by the presence of a very active NADPH oxidase. However, NADPH-dependent DTNB reduction could be demonstrated after preliminary fractionation of the crude extract.

Cells of L. leichmannii were grown and harvested as previously described (Blakley, 1965). The washed cells, after suspension in phosphate buffer, were disrupted in a Sorvall Ribi Cell Fractionator, and the debris was removed by centrifugation. Nucleic acids, together with some protein, were removed by precipitation with streptomycin sulfate. Additional protein was removed by acid precipitation at pH 5 and, after neutralization of the supernatant, by heating at 60° for 5 minutes. The resulting supernatant catalyzed DTNB reduction by NADPH, indicating the presence of both thioredoxin and thioredoxin reductase activities. After ammonium sulfate fractionation, the two activities were separated by gel filtration on Sephadex G-100. Purified E. coli thioredoxin was used to detect fractions containing thioredoxin reductase activity by the DTNB assay. The enzyme fractions thus identified were then used to detect thioredoxin activity. Column

chromatography on DEAE cellulose resulted in further purification of the separated thioredoxin and thioredoxin reductase of L. leichmannii. Finally, the isolated proteins were concentrated by lyophilization (thioredoxin) and by elution from DEAE cellulose with concentrated buffer (thioredoxin reductase).

The thioredoxin preparation contained 1.75 mg protein/ml, and the thioredoxin reductase had a protein concentration of 0.22 mg/ml. Molecular weights of the two preparations were determined by the gel filtration method (Andrews, 1964), and values of the order of 12,000 and 55,000 were obtained for thioredoxin and thioredoxin reductase, respectively. One ml of the thioredoxin preparation oxidized 38 mμmoles of NADPH in the presence of the reductase. On the assumption that each mole of NADPH reduces one mole of thioredoxin, as has been shown for E. coli (Laurent et al., 1964), the thioredoxin preparation from L. leichmannii was 26% pure on the basis of the above molecular weight.

The two isolated protein fractions of L. leichmannii, when combined, catalyzed the reduction of DTNB by NADPH. The activities of the thioredoxin system of L. leichmannii and E.coli in DTNB reduction are compared in Table I. The results shown in Exp. 2 and 3 demonstrate that thioredoxin reductase of L. leichmannii can reduce E. coli thioredoxin at approximately half the rate of its natural substrate. On the other hand, the thioredoxin reductase of E. coli, while showing high activity with its natural substrate (Exp. 1), reduces L. leichmannii thioredoxin only at a negligible rate (Exp. 4). Thus the two thioredoxin reductases exhibit a marked difference in substrate specificity. The difference in rate of the two complete systems (Exp. 1 and 2) is of no significance as the reductases were of unknown molarity.

TABLE I

DTNB reduction by the thioredoxin systems of E. coli and L. leichmannii

Exp.	E. coli		L. leichmannii		DTNB reduction (mμmoles/min)
	Thioredoxin (mμmoles)	Thioredoxin reductase (μg)	Thioredoxin (mμmoles)	Thioredoxin reductase (μg)	
1	0.4	5			87
2			0.4	5	131
3	0.4			5	58
4		5	0.4		2

The reduction of DTNB was measured at 412 mμ on a Cary Model 15 recording spectrophotometer. The experimental curvette contained 200 μmoles of potassium phosphate buffer, pH 7.5, 10 μmoles of EDTA, 80 mμmoles of DTNB, 100 mμmoles of NADPH, thioredoxin and thioredoxin reductase as indicated in a total volume of 1.0 ml. The reference cuvette contained an identical mixture, except for the omission of either thioredoxin or thioredoxin reductase.

Fig. 1 shows the rates of GTP reduction by the ribonucleoside triphosphate reductase at various non-saturating concentrations of thioredoxin from either E. coli or L. leichmannii. Each thioredoxin was tested in presence of excess thioredoxin reductase from the same organism. Under these conditions, reaction (1) was much faster than reaction (2) so that practically all of the thioredoxin was in the reduced form, and it was therefore possible to determine the K_m for the reduced thioredoxin in reaction (2). The system exhibited simple Michaelis-Menten kinetics with thioredoxin from either source. The apparent K_m for L. leichmannii thioredoxin was 3×10^{-6} M, and for E. coli thioredoxin 4×10^{-6} M. The slight difference in the apparent V_{max} obtained under the experimental conditions employed (Fig. 1B) is probably not significant.

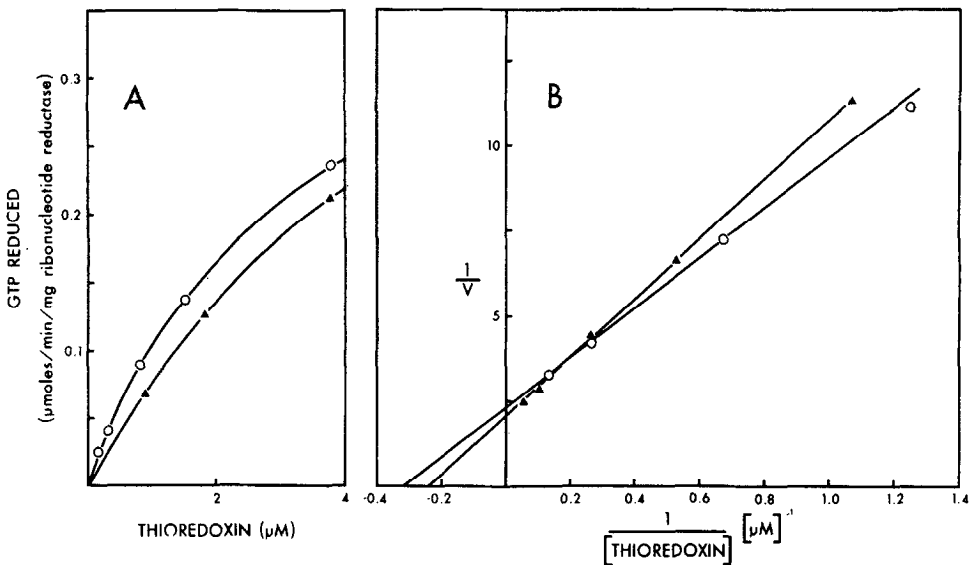

Fig. 1. The rate of GTP reduction as a function of thioredoxin concentration. The rate of the ribonucleotide reductase reaction was assayed at 37° by measuring NADPH oxidation at 340 mμ on a Cary Model 14 recording spectrophotometer. The experimental cuvette contained 100 μmoles of potassium phosphate buffer, pH 7.5, 2 μmoles of EDTA, 1 μmole GTP, 100 mμmoles NADPH, 15 μg of L. leichmannii ribonucleoside triphosphate reductase, 5 or 10 μg of thioredoxin reductase, 0.16 - 18.8 μM thioredoxin and 2 mμmoles 5'-deoxyadenosylcobalamin in a total volume of 0.5 ml. The reaction was started by the addition of the cobamide coenzyme after a 3 min. preincubation. The content of the reference cuvette was identical except for the omission of GTP. o——o thioredoxin system of L. leichmannii; ▲——▲ thioredoxin system of E. coli.

The protein fractions of the thioredoxin system of L. leichmannii still contained some NADPH oxidase activity after isolation. This activity was barely measurable in the thioredoxin fraction (5 mμmoles/min/mg), but it was appreciable in the thioredoxin reductase (345 mμmoles/min/mg). Attempts to separate this contaminating activity on hydroxylapatite columns have so far been unsuccessful. Electrophoresis on polyacrylamide gel (Ornstein and Davis, 1962) at pH 8.6 showed two closely associated bands for the thioredoxin preparation (Fig. 2).

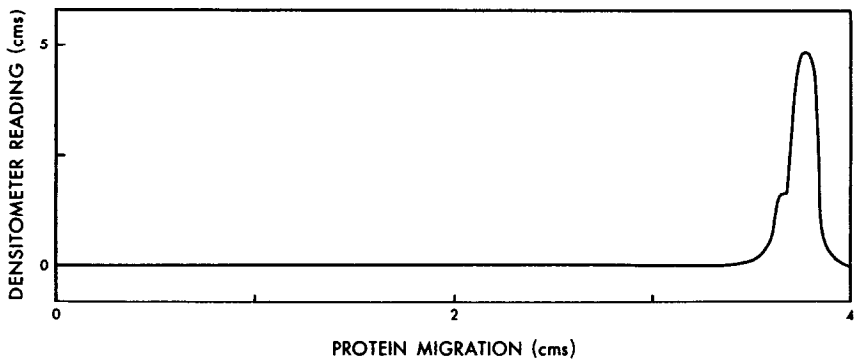

Fig. 2. Polyacrylamide gel electrophoresis of the thioredoxin fraction. The column was photographed, and the negative was scanned in a Spinco Analytrol Model RA Densitometer.

It is tentatively assumed that the smaller band, which appears as a shoulder in Fig. 2, represents thioredoxin, since the concentration of this protein is approximately the same as that calculated by NADPH oxidation (26%). Electrophoresis of the thioredoxin reductase under identical conditions produced seven separate bands indicating that this preparation was still grossly contaminated.

The total yield of thioredoxin from 150 g of wet cell paste was 0.8 μmoles. Even without allowing for losses during isolation, it can be calculated that this amount of thioredoxin is more than capable of supporting the total synthesis of deoxyribonucleotides in the organism. We therefore conclude that this thioredoxin system is the most likely physiological hydrogen donor for the ribonucleoside triphosphate reductase of L. leichmannii. Further study on the purification and detailed characterization of these two proteins is in progress.

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