A THIOREDOXIN - THIOREDOXIN REDUCTASE SYSTEM FROM RAT TUMOR*

E. Colleen Moore

Department of Biochemistry
The University of Texas M. D. Anderson
Hospital and Tumor Institute
Houston, Texas 77025

Received August 28, 1967

The reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides by the ribonucleotide reductase system of Escherichia coli is linked to TPNH[1] by thioredoxin and thioredoxin reductase (Laurent, Moore & Reichard, 1963; Moore, Reichard & Thelander, 1963). Thioredoxin is a small (12,500 m.w.) protein which is oxidized from a dithiol to a disulfide during ribonucleotide reduction, and regenerated by TPNH and the flavoprotein thioredoxin reductase. A similar system has been described in extracts of Lactobacillus leichmanii (Orr and Vitols, 1966). A requirement for a protein factor (Enzyme S) linking ribonucleotide reduction to TPNH has been described for the system from Novikoff rat tumor (Moore & Hurlbert, 1962; Moore & Reichard, 1963), but the components of this system have not previously been adequately characterized. This communication will report the separation and partial purification of two fractions from the Enzyme S preparation which correspond to the thioredoxin and thioredoxin reductase of E. coli. Following

*This investigation was supported by research grant CA 04464 from the National Cancer Institute.

[1]The following abbreviations are used: TPN - triphosphopyridine nucleotide; TPNH - reduced triphosphopyridine nucleotide; Tris - trishydroxymethylaminomethane; EDTA - ethylenedinitrilotetraacetic acid; ATP - adenosine triphosphate; CDP - cytidine diphosphate; P^{32}-CDP - cytidine diphosphate-α-P^{32}; FAD - flavin adenine dinucleotide; DEAE - diethylaminoethyl.

the example of Orr and Vitols (1966), I will use thioredoxin and thioredoxin reductase as generic terms for similar but not necessarily identical proteins from any organism having the same functions as the factors from E. coli.

Assays

During purification the fractions were assayed for stimulation of reduction of P^{32}-CDP by the partially purified nucleotide reductase system from E. coli (Factor B, Reichard, 1962) in the presence of TPNH. Each assay tube contained, in 0.12 ml, 1 μmole Tris-Cl buffer, pH 8.45, 0.08 μmole EDTA, 0.5 μmole ATP, 1.25 μmole magnesium acetate, 0.02 μmole P^{32}-CDP (approximately 1.2×10^6 cpm/μmole), 0.1 μmole reduced glutathione, 0.05 μmole TPNH, 0.05 μmole TPN, 0.09 μmole glucose 6-phosphate, 0.05 unit glucose 6-phosphate dehydrogenase, 0.0002 μmole FAD, and 0.13 mg nucleotide reductase from E. coli (containing an excess of thioredoxin reductase as a contaminant), plus an excess of one tumor fraction and a limiting amount of the other. Incubation was for 15 min at 38°.

The most purified fractions were also assayed with the nucleotide reductase (Enzyme P) from tumor, in a mixture containing 1 μmole potassium phosphate buffer pH 7, 0.25 μmoles ATP, 0.05 μmole magnesium acetate, 0.08 μmoles ferrous ammonium sulfate, and the other components (except EDTA) in the same amounts listed above. 0.2 mg nucleotide reductase was used and tubes were incubated 30 minutes. The isolation and counting of the product has been described (Reichard, 1962).

It was not possible to use the colorimetric assay for production of sulfhydryl groups in the presence of TPNH (Laurent, Moore & Reichard, 1963) because of very high blanks with one fraction alone.

Enzyme Purification

The neutralized pH 5 supernatant from the tumor extract (Enzyme S, Moore & Hurlbert, 1962) could completely replace thioredoxin in the assay with E. coli nucleotide reductase. After ammonium sulfate precipitation, however, two

fractions were required for activity. The fraction precipitating at 45-60% saturation, Enzyme S-B, after dialysis, was adsorbed on a column of calcium phosphate gel on cellulose, washed with 0.025 M phosphate, and eluted with 0.2 M phosphate. After concentrating by ultrafiltration, it was further purified by chromatography on DEAE-cellulose with a linear gradient approaching 0.5 M phosphate. Its specific activity was increased about 100-fold over the pH 5 supernatant. The fraction precipitating at 60-80% ammonium sulfate saturation (Fraction S-C) was purified by heat treatments at 67° and 75°, a second ammonium sulfate precipitation, and gradient elution from DEAE-cellulose. Its final specific activity was increased about 150-fold over the pH 5 supernatant.

Results

Table I illustrates the requirement for both purified fractions to replace thioredoxin when assayed with E. coli nucleotide reductase. Neither fraction alone gave any stimulation either at the relatively high concentration shown or at a lower concentration (not shown), but both together, even in the smaller amounts, gave an activity over half that seen with thioredoxin. In other

TABLE I

Requirements for Two Fractions with E. coli Nucleotide Reductase

Additions	Activity mμmoles dCDP
None	0.11
Thioredoxin	1.28
Enzyme S-B, 3 μg	0.11
Fraction S-C, 13 μg	0.13
Enz. S-B, 0.6 μg + Frac. S-C, 6.6 μg	0.80
Heated Enzyme P, 60 μg	0.14
Enz. S-B, 1.5 μg + Heated Enz. P, 60 μg	0.55

Each tube contained P^{32}-CDP, ATP, Mg^{++}, TPNH, TPNH generating system, glutathione and E. coli nucleotide reductase (containing thioredoxin reductase) in amounts listed under assays. Incubation was for 15 minutes. Enzyme S-B and Fraction S-C were the purest preparations described in the text. Heated Enzyme P was a tumor nucleotide reductase preparation inactivated by warming to 52°.

experiments, the activity obtained with S-B plus S-C at higher levels has fully equalled that with thioredoxin.

When the S-fractions were assayed with the tumor nucleotide reductase, the dependence on Fraction S-C was much less obvious (Table II). A test of the tumor nucleotide reductase (Enzyme P, heated) with the E. coli reductase showed considerable S-C activity (Table I). Thus it appears that just as the E. coli nucleotide reductase at this stage of purification is contaminated with thioredoxin reductase, the tumor nucleotide reductase is contaminated with Fraction S-C.

TABLE II

Requirement for Activity with Tumor Nucleotide Reductase

Addition	Activity mμmoles dCDP
Dithioerythritol	1.87
E. coli Thioredoxin, 0.18 mμmoles	0.08
E. coli Thioredoxin reductase, 0.4 μg	0.02
Thioredoxin + thioredoxin reductase	1.34
Enzyme S-B, 2.3 μg	0.45
Fraction S-C, 11.2 μg	0.05
Enz. S-B + Frac. S-C	0.59
Enz. S-B + thioredoxin	0.61

Each tube contained P^{32}-labeled CDP, ATP, Mg^{++}, Fe^{++}, TPNH, TPNH generating system, glutathione, and tumor nucleotide reductase (Enzyme P), in amounts listed under assays. Incubation was for 30 minutes.

While these fractions have not yet been completely characterized, it seems logical to assume on the basis of heat stability that Fraction S-C corresponds to thioredoxin and Enzyme S-B to thioredoxin reductase. Preliminary experiments (unpublished) with an earlier preparation similar to Enzyme S-B, assayed by stimulation of the tumor nucleotide reductase, indicated that precipitation with ammonium sulfate at pH 3 induced a dependence on added FAD. This further indicates its probable correspondence to thioredoxin reductase.

It appears that there is no cross reaction between the tumor thioredoxin and the thioredoxin reductase of E. coli. The nucleotide reductase preparation

from E. coli used in the assay contains an excess of thioredoxin reductase, so that the addition of thioredoxin alone is sufficient for activity. Thus the requirement for both tumor fractions indicates that the bacterial thioredoxin reductase cannot act on tumor thioredoxin. The experiment shown in Table II suggests that tumor thioredoxin reductase may be able to utilize E. coli thioredoxin, but the stimulation obtained either with thioredoxin or with Fraction S-C is not great enough to permit a definite decision on this point. Similar limited cross reaction with the thioredoxin-thioredoxin reductase systems from E. coli and from L. leichmanii has been reported by Orr & Vitols (1966).

Neither Enzyme S-B nor Fraction S-C is electrophoretically homogeneous at this stage of purification. Further purification is being attempted, and a more complete report will be presented later.

References

1. Laurent, T. C., Moore, E. C., & Reichard, P., J. Biol. Chem. 239: 3435-44 (1964).

2. Moore, E. C., & Hurlbert, R. B., Biochim. et Biophys. Acta 55: 651-663 (1962).

3. Moore, E. C., & Reichard, P., J. Biol. Chem. 239: 3453-56 (1964).

4. Moore, E. C., Reichard, P., & Thelander, L., J. Biol. Chem. 239: 3445-52 (1964).

5. Orr, M. D. & Vitols, E., Biochem. Biophys. Res. Comm. 25: 109 (1966).

6. Reichard, P., J. Biol. Chem. 237: 3513 (1962).