Biochimica et Biophysica Acta, 327 (1973) 274–281

BBA 67088

PURIFICATION AND CHARACTERIZATION OF YEAST THIOREDOXIN REDUCTASE

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(Received July 6th, 1973)

SUMMARY

Thioredoxin reductase (NADPH:oxidized-thioredoxin oxidoreductase, EC 1.6.4.5) from baker's yeast has been purified and some of its properties investigated. Yeast thioredoxin reductase is a flavoprotein with a molecular weight of approx. 75 000 and consists of two subunits, each containing one molecule of FAD.

The absorption spectrum is almost identical to the spectrum of the thioredoxin reductase from *Escherichia coli*, whereas large differences were found in the amino acid composition.

The molecular activity (moles of reduced triphosphopyridine nucleotide oxidized per min per mole of FAD) determined at 25 °C and pH 6.8 is 2000, at infinite concentration of thioredoxin; under these conditions a K_m value for thioredoxin of $4.4 \cdot 10^{-6}$ M was obtained.

Disulfide groups seem to play an essential role in the catalytic process.

INTRODUCTION

Thioredoxin reductase, a flavoprotein first isolated from *Escherichia coli*[1], catalyzes the NADPH-dependant reduction of thioredoxin, a small protein containing one disulphide bridge, which acts as an electron donor in the reduction of ribonucleotides to deoxyribonucleotides[2]. Thioredoxin systems from other sources as *Lactobacillus leichmanii*[3], Novikoff hepatoma[4], rat liver[5] and phage-infected *E. coli*[6] have been described.

Both thioredoxin and thioredoxin reductase were obtained in the pure form from *E. coli* B (refs 7 and 8).

Porqué *et al.*[9] reported a partial purification of baker's yeast thioredoxin reductase. We have attempted a further purification of the latter enzyme.

This paper described purification procedures, molecular weight, absorption spectra, FAD content and physico-chemical properties of the pure enzyme.

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PCMS, *p*-chloromercuriphenyl-sulphonate.

MATERIALS AND METHODS

DEAE-cellulose was the Whatman DE-32 type. NADPH and standard proteins for molecular weight determinations were from Boehringer, Mannheim, Germany; DTNB from Aldrich, Milwaukee, Wisconsin, U.S.A. All other chemicals were analtyical grade.

Baker's yeast was suspended in half its volume of 0.05 M Tris buffer (pH 7.5) and disintegrated in a refrigerated mill, using glass beads 0.5–0.7 mm in diameter (100 g/100 ml of yeast suspension). Disintegration was effected twice for 2 min with an interval of 2 min for cooling. The homogenate was diluted with 2 vol. of Tris buffer and stirred for 1 h. Insoluble material was removed by centrifugation (20 min) at 15 000 × *g*. The purification was carried out until the Sephadex G-100 step essentially as described by Porqué *et al.*[9]. The active fractions from Sephadex G-100 were concentrated 10-fold by ultrafiltration, and chromatographed on a column of DEAE-Sephadex A-50 (15 cm × 86 cm) equilibrated with 0.05 M Tris buffer (pH 7). Elution was effected with a linear gradient of Tris buffer (pH 7) (0.05–0.3 M, 200 ml each) at a flow rate of 12 ml/h. The main active fractions were concentrated by ultrafiltration, dialyzed against 0.02 M Tris buffer (pH 7) and rechromatographed on a column of DEAE-Sephadex A-50 (0.9 cm × 58 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of Tris buffer (pH 7) (0.2–0.3 M, 150 ml each) at a flow rate of 6 ml/h. The 3-ml fractions which showed the maximal specific activity, were pooled, concentrated by ultrafiltration, and dialyzed against 0.1 M potassium buffer (pH 7). After dialysis the enzyme was used for the experiments as described below.

Thioredoxin was purfied according to Porqué *et al.*[9] until the first DEAE-cellulose chromatography step. Fractions corresponding to the second peak of activity (thioredoxin II) were collected, lyophilized, redissolved in water and used to test thioredoxin reductase activity.

During the enzyme purification the protein content of the thioredoxin reductase was determined by reading the absorbance at 280 nm. The same procedure was used for the protein content of thioredoxin. One $A_{280\ nm}$ unit is defined as the amount of protein per ml that gives an absorbance of 1.00 at 280 nm. Alternatively for the thioredoxin the method of Lowry *et al.*[10] was used.

Thioredoxin reductase was assayed routinely by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) method as described by Porqué *et al.*[9] using 21.7 μg of thioredoxin (after chromatography on DEAE cellulose) per assay cuvette. 1 unit of reductase activity is defined as the amount of enzyme that gives a change in absorbance at 412 nm of 1.0 per min at 25 °C. Specific activity is defined as enzyme units per $A_{280\ nm}$ unit of protein. Alternatively the enzyme activity was tested following the decrease in absorbance at 340 nm (oxidation of NADPH) at 25 °C. The latter method was used for the kinetic studies and the inhibition by mercurials.

Disc gel electrophoresis was performed on 7.5% acrylamide gels in Tris–glycine buffer (pH 8.3) according to the procedure of Davis[11]. Acrylamide gel electrophoresis in sodium dodecylsulphate was performed according to the method of Weber and Osborn[12].

The molecular weight of the native enzyme was determined by thin-layer chromatography on Sephadex G-200, using the "sandwich technique" as described by Determan and Maettner[13].

Spectra were recorded with a Cary Model 14 recording spectrophotometer.

The amino acid analyses were carried out on a Beckman 120 B analyzer according to Moore and Stein[14]. Corrections for the destruction of threonine, serine and for the incomplete hydrolysis of valine and isoleucine were determined by hydrolyzing the samples for 24 and 72 h. Cysteine *plus* cystine content was determined as cysteic acid after oxidation with dimethylsulphoxide according to the method of Spencer and Wold[15]. The cysteic acid was calculated by reference to the amount of the stable amino acids (aspartic and glutamic acid, glycine, alanine and leucine).

RESULTS AND DISCUSSION

Purification of thioredoxin reductase

The purification was carried out as outlined in Table I.

TABLE I

PURIFICATION OF YEAST THIOREDOXIN REDUCTASE

Fraction	*Volume (ml)*	*Protein ($A_{280\ nm}$ units)*	*Activity (total units)*	*Spec. act. (units/$A_{280\ nm}$ unit)*
Sephadex G-50	1964	8612	5161	0.6
DEAE-cellulose	13.5	249	1940	7.7
Sephadex G-100	8.5	59	1600	27
I Sephadex A-50	5	15	1342	90
II Sephadex A-50	0.8	4.6	485	103

The sharp protein peak on the elution profile of the 2nd Sephadex A-50 column superimposes the activity peak (Fig. 1). The most active fractions were eluted at 0.25 Tris buffer and showed a constant specific activity of 103. The homogeneity of this

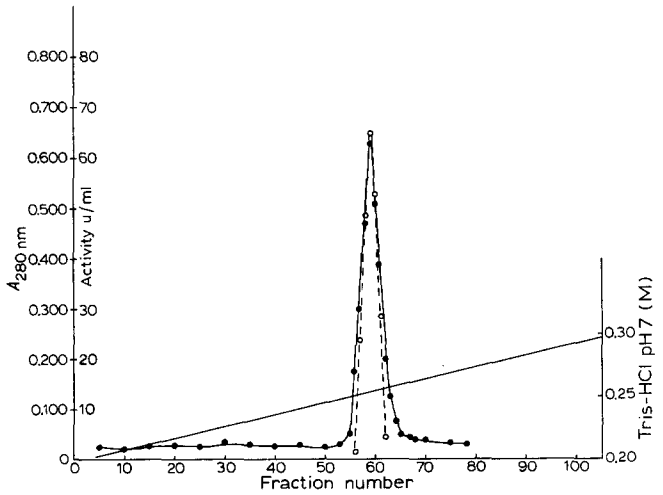

Fig. 1. Elution profile from the second Sephadex A-50 chromatography. ●—●, $A_{280\ nm}$; ○—○, enzyme activity.

material was tested by disc electrophoresis on polyacrylamide gel (Fig. 2a): the gel was overloaded with 80 μg of protein and a single band was obtained. Disc electrophoresis with sodium dodecylsulphate also gave a single band (Fig. 2b).

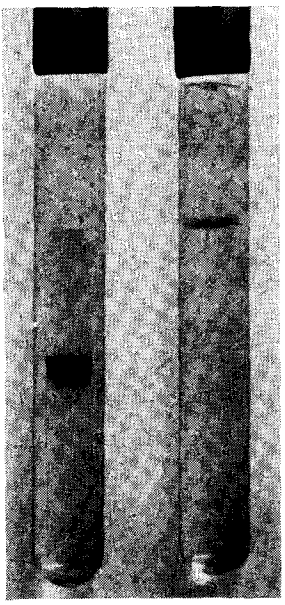

Fig. 2. Disc electrophoresis of yeast thioredoxin reductase after the second Sephadex A-50 chromatography. (a). Analytical disc electrophoresis (80-μg sample). (b). Disc electrophoresis in sodium dodecylsulphate (15-μg sample).

Molecular weight

Sodium dodecylsulphate disc electrophoresis gave a molecular weight of 38 000 (Fig. 3) for the enzyme monomer. In order to know the degree of aggregation, if any, the molecular weight of the native yeast thioredoxin reductase was also deter-

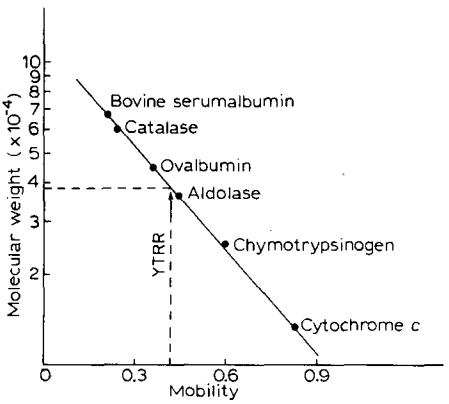

Fig. 3. Molecular weight determination of yeast thioredoxin reductase (YTRR). Plot of molecular weight *vs* relative mobility to the tracking dye on sodium doedcylsulphate disc electrophoresis.

mined by thin-layer gel filtration on Sephadex G-200 and a value of 75 000 was obtained (Fig. 4) which is indicative of a dimeric enzyme, consisting of two polypeptide chains.

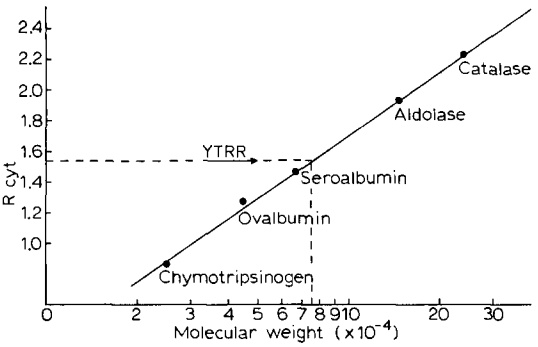

Fig. 4. Molecular weight determination of yeast thioredoxin reductase (YTRR) by thin-layer gel filtration on Sephadex G-200. Plot of molecular weight *vs* protein migration related to cytochrome *c* mobility.

Spectrum and FAD content

The spectrum of thioredoxin reductase, shown in Fig. 5 has absorption maxima at 380 and 456 nm, minima at 320 and 408 nm and a shoulder at 480 nm.

The ratio $A_{280\ nm}/A_{450\ nm}$ of the pure enzyme is 6.7. After boiling the enzyme for 5 min the spectrum of free FAD was obtained with maxima at 373 and 450 nm. by applying an $\varepsilon = 11\ 300\ M^{-1} \cdot cm^{-1}$ for the enzyme-bound FAD, at 456 nm (ref. 16) a content of 12.9 nmoles of FAD has been calculated per 1 $A_{280\ nm}$ unit of enzyme.

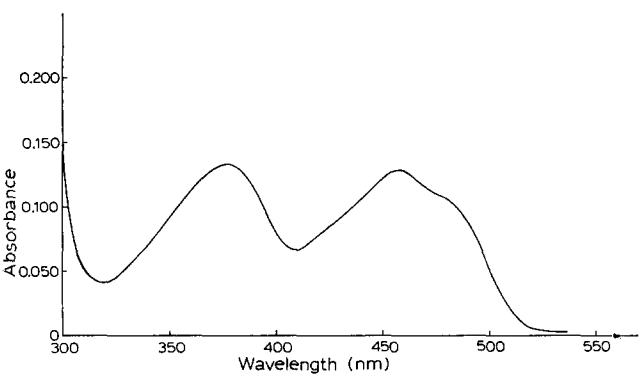

Fig. 5. Spectrum of native yeast thioredoxin reductase obtained with a solution of thioredoxin reductase after the second DEAE-Sephadex A-50 chromatography (0.89 $A_{280\ nm}$ units per ml of 0.1 M phosphate buffer (pH 7)).

Amino acid analysis

The amino acid composition of thioredoxin reductase is reported in Table II. Yeast thioredoxin reductase seems to be quite different in amino acid composition from the *E. coli* enzyme[16–18] especially for the content of the basic amino acids. On

TABLE II

AMINO ACID COMPOSITION OF YEAST THIOREDOXIN REDUCTASE

Amino acid	*Moles/mole FAD*
Asp	28.5
Thr	27.5
Ser	20.4
Glu	32.9
Pro	13.4
Gly	29.1
Ala	34.5
Cys*	4.6
Val	18.7
Met	6.6
Ile	20.4
Leu	26.0
Tyr	8.5
Phe	11.4
Lys	24.2
His	5.5
Arg	10.9
Total residues	323

* Determined as cysteic acid after dimethylsulphoxide oxidation.

the contrary a content of 4.6 half-cystine residues per FAD, determined as cysteic acid after dimethylsulphoxide oxidation, has been calculated, as in the case of the *E. coli* enzyme. The molecular weight which results from the amino acid composition, not taking into account the tryptophan content, is of 34 500 per FAD. The value is in good agreement with the molecular weight of 38 000 found by sodium dodecylsulphate gel electrophoresis.

Molecular activity and K_m determination for thioredoxin

The molecular activity of fresh purified thioredoxin reductase, determined at 25 °C in 0.1 M potassium phosphate buffer (pH 6.8) at an infinite concentration of thioredoxin, corresponds to 2000 moles of NADPH oxidized per min/mole of FAD; in these conditions a K_m value for thioredoxin II of $4.4 \cdot 10^{-6}$ M was obtained.

Evidence for the presence of a disulphide at the catalytic site

The data reported in Table III show the effect of a low concentration of *p*-chloromercuriphenylsulphanate (PCMS) on the enzyme activity. The enzyme was preincubated with PCMS alone or with PCMS and NADPH: preincubation of thioredoxin reductase with PCMS has practically no effect on the activity, whereas PCMS + DANPH in the preincubation mixture causes a complete inhibition. Control preincubation with NADPH alone does not effect the activity. The results are similar to those obtained for the *E. coli* enzyme[17,19] indicating that the inhibition by the mercurial is due to the titration of the nascent dithiol, arising from the active centre disulphide produced by the added substrate (NADPH).

The present study shows that yeast thioredoxin reductase consists of two identical or very similar subunits of a mol. wt of approx. 38 000. The evidence comes from the results of acrylamide gel electrophoresis in sodium dodecylsulphate, from the

TABLE III

EFFECT OF PREINCUBATION WITH NADPH ON THE SENSITIVITY OF YEAST THIOREDOXIN REDUCTASE TO PCMS

Assays were carried out at 25 °C in 1 ml of final volume containing 0.1 μmole of NADPH, 100 μmoles of potassium phosphate buffer (pH 6.8), 10 μmoles of EDTA, 6.4 nmoles of thioredoxin, thioredoxin reductase corresponding to 0.0025 nmole of FAD were preincubated in the assay cuvette for 2 min and the reaction was started by the addition of the substrates. The activity is expressed as decrease in absorbance at 340 nm per min.

Preincubation conditions	*Activity*
Control	0.0127
NADPH	0.0127
PCMS (0.5 μM)	0.0118
PCMS (0.5 μM) + NADPH	0.0000

molecular weight determination of the native enzyme on thin-layer chromatography and from the amino acid content calculated on the basis of 1 FAD molecule. The above results are thus consistent with an enzyme which contains 2 moles FAD/per mole of enzyme of a mol. wt of approx. 75 000.

The amino acid analysis reveals large discrepancies in the amino acid composition between the thioredoxin reductase from *E. coli* and the same enzyme extracted from yeast. On the contrary an identical content of half-cystine was found for both the enzymes.

The spectral and kinetic properties of the yeast enzyme are very similar to those of the *E. coli* enzyme. The kinetic results obtained in the yeast enzyme after inhibition by mercurials seem to indicate that a catalytic mechanism involving a disulphide group could be postulated as for the enzyme extracted from *E. coli*.

ACKNOWLEDGEMENTS

This work was supported by a NATO Grant (No. 439) and by a grant from the Italian Research Council (No. 71.00805.05).

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