PAPS-Reductase from *Escherichia coli:* Characterization of the Enzyme as Probe for Thioredoxins

Jens D. Schwenn and Ulrich Schriek

Biochemie der Pflanzen, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum 1, Bundesrepublik Deutschland

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PAPS-reductase from *Escherichia coli* was employed to detect thioredoxins from pro- and eukaryotic organisms. A simple method for the isolation of this enzyme and properties of the enzymatic assay were described. A comparison between thioredoxins detected by the PAPS-reductase and the Fructose-bisphosphatase or NADP malate dehydrogenase was used to assess the validity of the test. The high cross-reactivity of the bacterial enzyme was useful in the purification of heterologous thioredoxins from spinach, *Synechococcus,* and *Saccharomyces cerevisiae.*

Introduction

Thioredoxins are multifunctional redox proteins which contribute to the regulation of enzyme and receptor activity, to hydrogen transfer and also to the assembly of phages (survey [1]). Since thioredoxins lack own catalytic properties usable for their detection a specific enzyme reaction is needed to demonstrate the presence of a thioredoxin. Widely accepted methods are:

a) enzyme activation (thioredoxin is "non-essential" for enzymic catalysis but modulates activity),
b) ribonucleotide reduction (thioredoxin is used as cosubstrate by the ribonucleoside reductase), and
c) protein disulphide reduction (thioredoxin is used as disulphide reductase for insulin A–B chain reduction).

Since thioredoxin-activated enzymes of the C-autotrophs like plants have developed a specificity for an individual thioredoxin, this specificity is currently used as basis for classifying the different plant thioredoxins [2]. Yet, this specificity makes these enzymes less suitable as general probe for other thioredoxins. Similarly, antibodies that have been raised against a well characterized thioredoxin risk to fail as indicator because evolutionary more distant thioredoxins may show little or no cross-reaction (*cf.* [3]). The more general characterization of a thioredoxin is based on its function as hydrogen donor in the reduction of ribonucleotides by the NADPH dependent ribonucleotide reductase(s) (EC 1.17.4.1, 1.17.4.2) [4, 5]. As for method c), a purified NADPH : thioredoxin oxidoreductase is the prerequisit of this NAPDH-dependent insulin chain A–B disulphide reduction [6]. Since the thioredoxin oxidoreductase is highly specific for the thioredoxin [7] it has been replaced by dithiothreitol, a non-physiological reductant, facilitating the determination of thioredoxins independent of the reducing enzyme system [8].

In this paper, we describe a method for the detection of thioredoxins which is based on their function as cosubstrate in the reduction of activated sulphate to sulphite [9, 10], according to:

$$PAPS^{-4} + Thio(SH)_2 \rightarrow HSO_3^- + Thio(S)_2 + PAP^{4-} + H^+.$$

^{35}S labelled PAPS has been used as substrate so that $[^{35}S]SO_3$ was formed as reaction product. Crude preparations of this enzyme have previously been shown to require thioredoxin as reductant [10, 11]. In addition, cross-reaction of the enzyme from *E. coli* with thioredoxin from HeLa cells [12] was reported. Still, methods for the purification of PAPS-reductase and details of the reaction are fragmentary. It, therefore, seemed worthwile to investigate this reaction in more detail using a purified PAPS-

Abbreviations: APS, adenylylsulphate; DTT, dithiothreitol; EDTA, ethylendiamine tetra acetic acid; FbPase, fructose-1,6-bisphosphatase; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; MDH, malate dehydrogenase; PAPS, 3′-phospho adenylylsulphate; 3′,5′-PAP, adenosine-3′,5′-bisphosphate; PMSF, phenylmethanesulphonyl fluoride; SDS PAGE, Na dodecylsulphate polyacrylamide; gel electrophoresis.

Reprint requests to Dr. J. D. Schwenn.

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reductase. A simple reliable method for the isolation of PAPS-reductase from *Escherichia coli* was developed with the aim to render the protein free of thioredoxin. The enzyme was characterized with emphasis on its substrate requirements. It was then used to detect thioredoxins from *E. coli, Synechococcus spec.**, *Saccharomyces cerevisiae*, spinach leaf material and rabbit liver. Heterologous thioredoxins from the different organisms were tested as cosubstrates in the reduction of PAPS investigating the enzyme's specificity for thioredoxin. The reliability of the method was compared with the established methods of thioredoxin detection (*e.g.* activation of NADP malate dehydrogenase or Fru-1,6-P phosphatase).

Materials and Methods

Thioredoxins

Thioredoxins were isolated from *Escherichia coli* (strain AN1460), *Saccharomyces cerevisiae* (commercial baker's yeast), a thermophile cyanobacterium (*Synechococcus spec.***), spinach leaf extract and rabbit liver. *E. coli* and *Synechococcus spec.* were disrupted in a French press (at 14 MPa) [13], yeast was extracted by auto-lysis with organic solvents [14], while rabbit liver and spinach leaves were homogenized in a Waring Blendor. PMSF (1 mM) was included as protease inhibitor. Crude protein extracts were obtained by centrifugation at $15000 \times g$ for 30 min, particulate contaminants were then removed by precipitation with ammonium sulphate (30% saturation at 4 °C). The protein was clarified by centrifugation as before and precipitated with ammonium sulphate to give 80% saturation. The fraction of protein precipitating from 30–80% has been used as starting material for the isolation of thioredoxins or, for the preparation of the PAPS reductase.

The bulk of thioredoxin(s) was separated from the enzyme by inverse ammonium sulphate fractionation (see below). Thioredoxin containing fractions were salted out (80% saturation) and collected by centrifugation at $20000 \times g$ for 30 min. The protein was dissolved in Tris-HCl 50 mM (pH 7.8) to give appr. 30 mg/ml, and desalted on Sephadex G50 (3.2 × 63 cm, column equilibrated with the same Tris buffer). Thioredoxin containing fractions were pooled and heated for 5 min at 70 °C. Denatured protein was separated by centrifugation while the supernatant was diluted with distilled water to give a conductivity of 1–1.2 mS allowing the protein to be collected on TSK DEAE 650 (1.8 × 16 cm, equilibrated with Tris-HCl 5 mM (pH 7.8). The weakly bound thioredoxins were eluted by a gradient of Tris-HCl (pH 7.8) 5 to 50 mM with NaCl increasing from 0 to 200 mM in a total volume of 400 ml. Fractions containing thioredoxin were combined and purified further by chromatofocusing as described recently [13] and by FPLC using TSK DEAE 5PW (LKB Gräfelfing, FRG), Mono Q and Superose 12 (Pharmacia Freiburg, FRG).

Thioredoxin *m* from spinach has been separated from the *f*-type thioredoxins by passage through Blue Sepharose Cl 6B as described by Schürmann *et al.* [15] prior to FPLC purification on TSK DEAE 5PW and Mono Q. Rabbit liver thioredoxin has not been purified further than gel filtration on Sephadex G50.

Fructose-bisphosphatase was prepared according to Buchanan *et al.* [16] substituting Sephacryl S300 for Sephadex and DEAE TSK for DEAE cellulose. The assay of thioredoxin *f* was essentially the same as published by Schürmann *et al.* [15] except for Mg^{2+} which was lowered to 0.5 mM.

NADP malate dehydrogenase was purified from spinach leaf protein (30–80% precipitate as above) by gelfiltration (Sephacryl S300) and ion exchange (DEAE TSK 650S). Activation by thioredoxin *m* was measured as described in ref. [15].

Purification of the PAPS-reductase from E. coli

Extraction of the bacteria (200 g) was identical to the procedure published recently [13]. As for thioredoxins, the 30 to 80% precipitate of the ammonium sulphate fractionation was used for further steps. The protein slurry was allowed to settle on a flat bed of DEAE cellulose (2.0 cm) mounted in a wide column. It was eluted by applying a linear gradient of A: ammoniumsulphate 80% saturation at 4 °C in Tris-HCl 50 mM (pH 7.8) and B: glycerol 30% (v/v) in Tris as before. The most active fractions were combined and precipitated with solid ammonium sulphate (80% sat.); the protein was then collected by centrifugation, dissolved in ammonium sulphate 1 M, buffered with Tris-HCl 50 mM (pH 7.4), EDTA 0.1 mM and, DTT 5 mM. Undissolved protein was removed by centrifugation. The

* The organism was a kind gift by Dr. S. Katoh, Tokyo University.

** This strain is not yet indexed.

remaining protein was recovered from the supernatant by adsorption onto Phenyl Sepharose Cl 4B (1.8 × 18.3 cm, equilibrated with the ammonium sulphate-Tris-EDTA buffer). The column was developed using a gradient (volume 400 ml) of A: ammonium sulphate 1 M and B: glycerol 30% (v/v) buffered with Tris-EDTA as above. *E. coli* thioredoxin was scarcely retained by the column but emerged with the buffer front. The enzyme usually was eluted at 0.4 to 0.3 M of ammonium sulphate. Active fractions were concentrated by ammonium sulphate (80%) and stored as suspension (containing EDTA 0.1 mM) at 0 °C for up to 16 weeks without considerable loss of activity. Further purification was not required in order to demonstrate the thioredoxin requirement (see Table I).

Enzymatic assay and unit definition

Up to 5 μg of protein were incubated for 5 min* at 30 °C in an assay mixture containing: Tris-HCl 50 mM (pH 8.0), DTT 5 mM, EDTA 1 mM, [^{35}S]PAPS 10 μM (spec. activity 160×10^3 to 26×10^3 Bq/nmol), Na_2SO_3 20 mM and thioredoxin 0.5 to 10 μg in a total volume of 100 μl. The reaction was stopped by injection of 100 μl of acetone. The minivials were decapped and placed into 15 ml scintillation vials containing 0.5 ml trioctylamine; 0.3 ml of sulphuric acid (3 M) were added to the minivial in order to initiate the distillation of sulphite. The scintillation vials were then sealed and heated to 60 °C 45 min. This treatment was sufficient to release volatile sulphurdioxide quantitatively. After cooling to 0 °C, the inner reaction vessel was removed and 10 ml of scintillant were added to the trioctylamine. With appropiate corrections, the amount of sulphite formed during the assay was strictly correlated to the amount of enzyme (or thioredoxin). The unit of PAPS-reductase activity was defined as 1 μmol [^{35}S]sulphite formed x min^{-1} under the above conditions (e.g.: PAPS and thioredoxin "saturating"**).

[^{35}S]PAPS was prepared enzymatically from [^{35}S]sulphate (Radiochemical Centre Amersham, England) as described previously [13]. Contamination of PAPS by APS, 3′5′PAP and sulphate was kept below 10%. Purity was controlled by HPLC analysis as detailed in ref. [17]. Trioctylamine was purchased from Fluka (Neu-Ulm, FRG), analytical grade chemicals from Merck (Darmstadt, FRG). Isoelectric focusing was carried out an Servalyte Precotes (pH intervals 3–10 or 3–6, Serva Heidelberg, FRG) applying 1950 or 1730 Vh. Coomassie Brillant Blue R-250 has been used for staining of proteins in IEF and SDS-polyacrylamide gel electrophoresis. SDS PAGE was carried out on 12% gels according to Laemmli [18]. Protein was determined by the method of Bradford [19] using bovine serum albumin as reference. Except for TSK DEAE-650 (Merck Darmstadt, FRG), TSK DEAE 5 PW (LKB Gräfelfing, FRG) other chromatographic supports used for the purification of thioredoxins and PAPS-reductase were from Pharmacia (Freiburg, FRG). Commercially available software (Interactive Microware Inc., State College, PA 16801, USA) was used for fitting of data.

Results

A simple and reliable procedure for the isolation of a substantially enriched PAPS-reductase is given in Table I. The steps were chosen as to make the enzyme thioredoxin-dependent. With the purified enzyme only 1.3% of the apparent rate accounted for sulphite formation in the absence of additional thioredoxin. The bulk of thioredoxin was removed by the inverse fractionation with ammonium sulphate; the remaining fraction was then separated from the enzyme during hydrophobic chromatography on Phenyl-Sepharose. Inactivation of the enzyme by dilution was circumvented by addition of glycerol (30%); this additive also seemed to prevent protein aggregation due to high hydrophobicity of the enzyme protein; otherwise, the enzyme was rapidly inactivated.

The reaction kinetics was linear (Fig. 1) for 1 to 5 μg of enzyme protein. Irregular kinetics have been observed when the enzyme was added from a high concentration of salt directly into the reaction mixture. Therefore, the enzyme was preincubated for 30 min at 30 °C starting the reaction by addition of the substrate PAPS. Under these conditions, a negligible deviation from linearity was observed only above E × t of 300 μg min (inset Fig. 1) whereas inadequate sampling seemingly accounted for minute product formation at zero-time.

* The amount of enzyme should be small enough to keep product formation low because 3′5′ PAP is inhibitory (see Fig. 3).

** Kinetic constants and mechanism will be published separately.

Table I. Fractionation of PAPS-reductase. 1 U was defined as 1 μmol sulphite formed · min^{-1} from PAPS (Materials and Methods, part c).

Step	Vol [ml]	Protein [mg]	Concentration [U · μl^{-2}]	Specif. activity [U · mg^{-1}]	Total units
ammonium sulphate [30–80% sat]	50	2500	0.164	3.3 (95.6)[a]	8190
inverse fractionation[b]	200	1128	0.123	24.5 (6.6)	27639
Phenyl Sepharose CL 4B[c]	324	192	0.341	114.4 (1.3)	22038

[a] residual activity in the absence of additional thioredoxin in % activity at saturating concentrations of thioredoxin.
[b] activation presumably due to the use of glycerol and the removal of high salt from previous step.
[c] extrapolated from preparation using 1/6 of the original amount of protein.

At saturating concentrations of PAPS (app. K_M 8 μM), the enzyme exhibited a high affinity (app. K_M 2 μM) for the homologous thioredoxin (Fig. 2A, amount of enzyme varied). Hence, linearity of enzyme activity and concentration of thioredoxin was maintained up to 1 μM of thioredoxin (Fig. 2B, concentration of PAPS varied). Limitations of this thioredoxin assay were caused through inhibition of the PAPS-reductase by its reaction product 3′,5′-PAP (Fig. 3). 3′,5′-PAP seemed to behave like a mixed type inhibitor since K_m and V_{max} were lowered. The K_I obtained by slope replot (inset Fig. 3) was 25 μM in the presence of fixed saturating concentrations of thioredoxin when PAPS was varied. Sulphite, as the second reaction product had no effect upon the enzyme's activity (tested for concentrations up to 20 mM). Due to the high concentration of DTT (5 mM), oxidized thioredoxin could not have

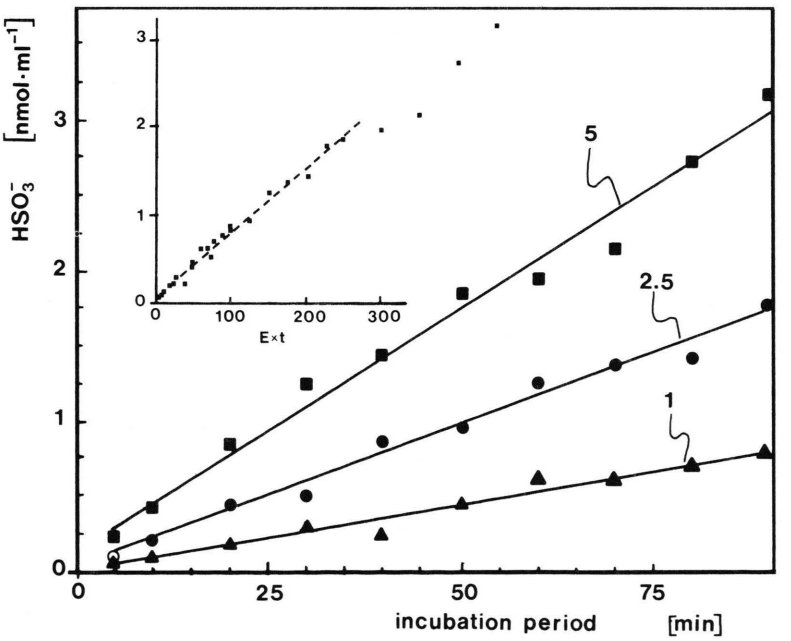

Fig. 1. Reaction kinetics of *E. coli* PAPS-reductase. Different amounts of enzyme (▲: 1 μg, ●: 2.5 μg, and ■: 5 μg protein) were incubated at intervals as indicated using saturating concentrations of homologous thioredoxin (23.85 μg/100 μl) and PAPS (16 μM). Inset in Fig. 1: Formation of sulphite as function of amount of enzyme × time [μg · min] extraplated from the same data. Reaction conditions as described in Materials and Methods, part (c).

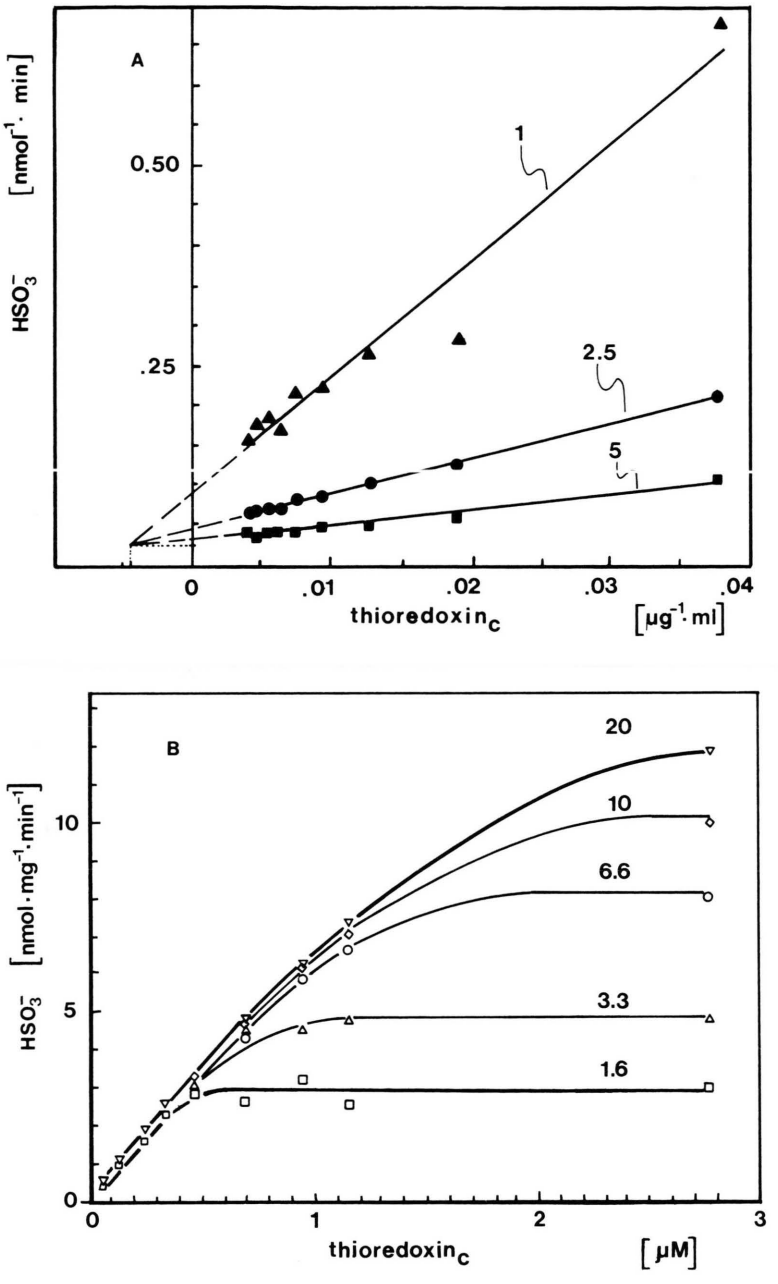

Fig. 2. Saturation of *E. coli* PAPS-reductase with homologous thioredoxin and PAPS. A: Rate of sulphite formation using different amounts of enzyme (▲: 1 µg, ●: 2.5 µg, and ■: 5 µg) as saturated by the homologous thioredoxin; app. K_M 2 µM determined from the common intercept of the double reciprocal plot. The concentration of PAPS was kept constant at 16 µM, equivalent to 2-times K_M PAPS as determined from data shown in Fig. 2B. B: Determination of the linear range of enzyme activity with respect to variations of the concentration of homologous thioredoxin and PAPS (amount of enzyme kept constant: 5 µg, concentrations of PAPS in µM and of thioredoxin varied as indicated, points below 0.5 µM of thioredoxin have been omitted for 10, 6.6 and 3.3 µM PAPS).

accumulated to any considerable amount and, therefore, appeared not relevant in this respect.

It has been noted previously, that the enzyme crossreacted with heat-labile proteins from HeLa cells which have thioredoxin- or glutaredoxin-like properties [12]. The relative contribution of heterologous thioredoxins as cosubstrate in the PAPS reductase test has been compared with the homologous system using the *E. coli* thioredoxin (Table II). The thioredoxin obtained from *Synechococcus*, supported PAPS reduction at a significantly higher rate (86%) than thioredoxins from spinach or yeast (54 to 50% of the bacterial cosubstrate). The data may indicate that the enzyme cross-

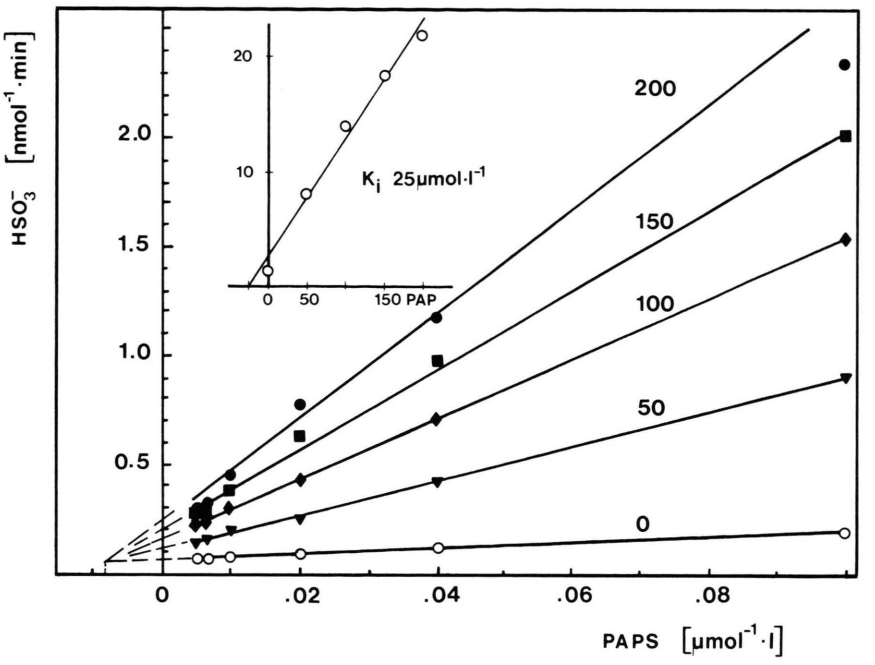

Fig. 3. Mixed type inhibition by 3′,5′-PAP of *E. coli* PAPS-reductase. PAPS as substrate was varied at fixed saturating concentrations of thioredoxin (23.85 µg/100 µl), concentrations of inhibitor (in µM) as indicated. Inset Fig. 4: Slope replot for determination of $K_{z,\ 3'5'PAP}$: 25 µmol/l.

reacts with evolutionary more distant thioredoxins with a lower efficiency than with the closer related bacterial thioredoxin.

Since the PAPS-reductase as characterized above combines high sensitivity and low specificity, the test system seemed particularly suited to monitor thioredoxin(s) from different species. We, therefore, compared this method with the thioredoxin-dependent enzyme activation in which Fructose-1,6-bisphosphatase and NADP malate dehydrogenase from higher plants serve as key enzymes. The distribution of thioredoxins from plant leaf extracts as detected by the PAPS-reductase from *E. coli* is depicted in Figure 4A. Two major fractions (design. I, II) supporting the PAPS-reductase were eluted at low ionic strength. These two fractions were non-homogeneous but split into several maxima indicating the presence of more than two thioredoxins. (Three minor fractions also occurred at NaCl > 95 mM, V_{eff} >250 ml, omitted from the figure). Using NADP malate dehydrogenase as an indicator enzyme (Fig. 4B), at least three thioredoxins *m* can be detected; all of which coelute with the thioredoxins as detected by the PAPS-reductase. If FbPase is used as probe, two thioredoxins *f* were detected of which only the late eluting thioredoxin coeluted with the thioredoxins of the PAPS-reductase test. The fast eluting thioredoxin (V_{off} 105 ml, fraction 52–56) was only marginally supporting the PAPS-reductase, if at all. It seems noteworthy, that this thioredoxin *f* did also not crossreact in the MDH assay for thioredoxin *m*.

Since the PAPS-reductase assay for thioredoxin indicated the presence of possibly more than three thioredoxins in fraction II as detected by the MDH

Table II. Cross-reactivity of *E. coli* PAPS-reductase with thioredoxins purified from different organisms. Homogeneous thioredoxins (5 µg) were assayed using DTT as reductant in the PAPS-reductase assay (1.5 µl of enzyme after hydrophobic chromatography on Phenyl-Sepharose) as described in Materials and Methods. Isoelectric points of the thioredoxins are given in brackets; rabbit liver thioredoxin was not purified to homogeneity.

Source of thioredoxin	Enzyme activity [nmol SO_3 min^{-1}]	Relative rate [%]
(a) *E. coli* (4.45)	2.2	100
(b) spinach (4.68)	1.10	50
(c) spinach (4.70)	1.24	54
(d) spinach (5.05)	1.3	59
(e) yeast (4.8/5.2)[a]	1.22	54
(f) *Synechococcus* (4.8)	1.90	86
(g) rabbit liver (crude)	0.0425	2

[a] Homogeneous by SDS-PAGE only; consisting of two proteins with pI 4.8 and pI 5.2 in IEF.

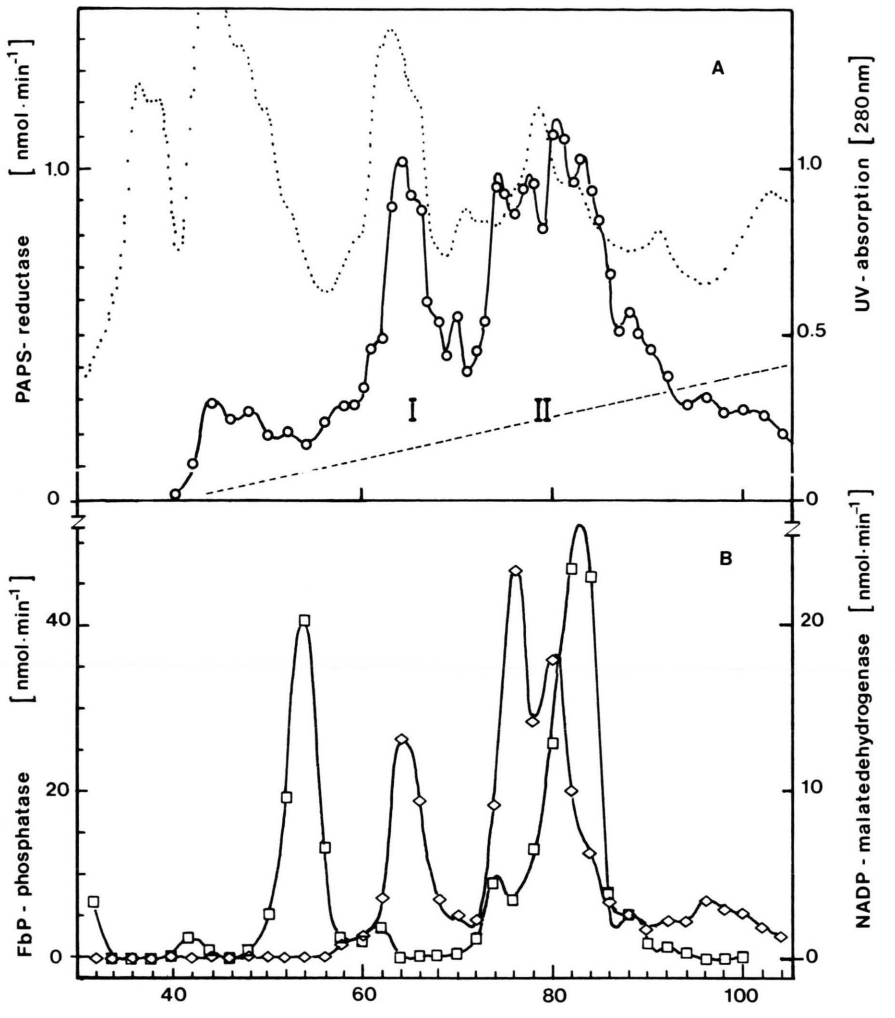

Fig. 4. Thioredoxins from spinach leaf protein as detected PAPS-reductase Fructose-bisphosphatase and NADP malate dehydrogenase as probes. A: The plant protein (2645 mg derived from 30–80% ammonium sulphate precipitate as described in Material and Methods) was separated on a column of TSK DEAE 650 S (size of fraction 2.5 ml, flow rate 48 ml/h, linear gradient of NaCl from 0 to 1 M, 0.1 M/100 ml, as indicated by dashed line). Content of thioredoxin was determined by using aliquots of 20 µl/fraction tested in the PAPS-reductase assay (enzyme protein from Phenyl Sepharose 7.76 µg/test, *e.g.* "excess" enzyme vs. thioredoxin, saturating PAPS 32 µmol/l). Distribution of thioredoxins obtained by extrapolation using cubic spline method (○—○), protein at 280 nm (·····). B: Distribution of thioredoxin *f* and *m* as indicated by the activation of FbPase (□—□) and MdH activity (◇—◇) assayed according to ref. [15], aliquots of 30 µl/fraction were used for the MDH- and 50 µl for the FbPase test.

and FbPase test, the corresponding fraction from a parallel preparation was used for further purification employing the PAPS-reductase assay. Thioredoxin *f* was removed by passage through a column of Blue Sepharose CL 6B (data not shown) which was observed to bind thioredoxin *f* [15] while the remaining thioredoxins were isolated by FPLC on DEAE TSK 5PW and MonoQ 5/5 (Fig. 5). Chromatography on this ion exchanger separated three thioredoxins; a fast eluting protein (R_t 10 min, Fig. 5I) and two more strongly retained proteins (R_t 16 and 19 min, Fig. 5II). The pI as obtained by chromatofocusing (Fig. 6) of the fast eluting thioredoxin was 5.05. The late eluting thioredoxin fractions were more acidic (pI 4.7 and 4.68). Isoelectric focusing of the thioredoxins isolated by this method confirmed their

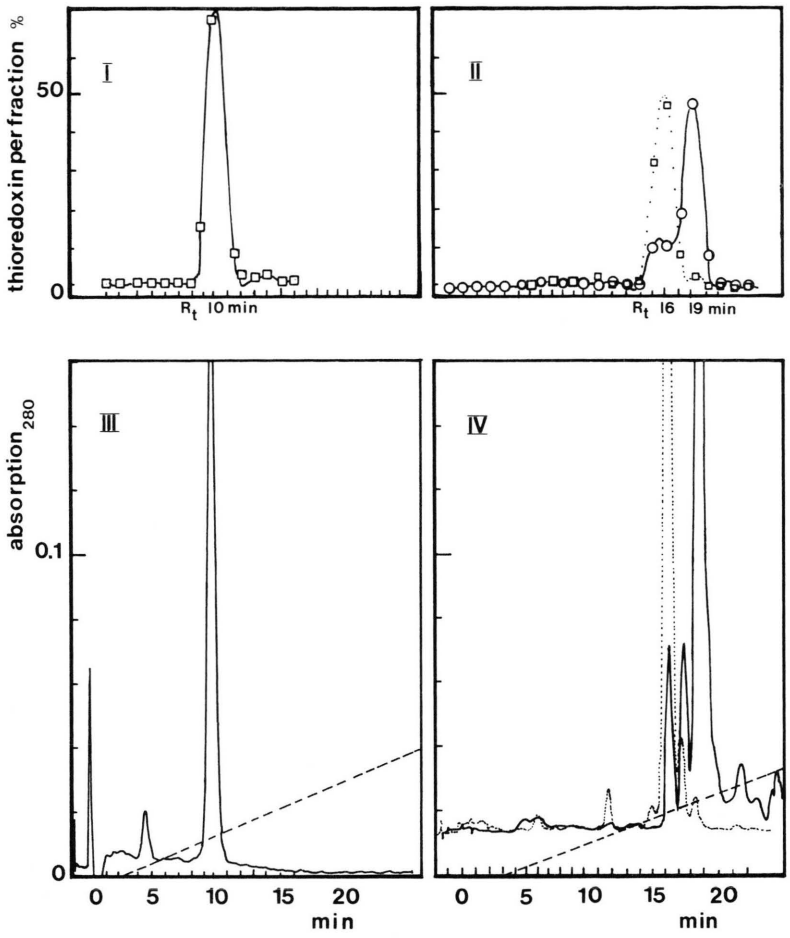

Fig. 5. Distribution of spinach leaf thioredoxins after FPLC separation on MonoQ ion exchanger. Fig. 5I: thioredoxin pI 5.05, Fig. 5II: thioredoxins pI 4.70 (lane 5 in Fig. 6) and pI 4.68 (lane 6 in Fig. 6). Thioredoxins (originated from fraction II after rechromatography on TSK DEAE 5PW and removal of thioredoxin *f* by chromatography on Blue Sepharose CL 6B) were separated on a column of MonoQ 5/5, flow rate 1.5 ml/min, eluent A: Tris-HCl 25 mM (pH 7.8), eluent B: Tris buffer including NaCl 1 M, lin. gradient 1 mM/1ml (– – – –) PAPS-reductase was used as indicator enzyme using aliquots of 50 µl/fraction as source of thioredoxin, other reaction conditions as described in the legend to Fig. 4, R_t: retention time in [min], distribution evaluated by cubic spline. Fig. 5 III and Fig. 5IV: absorbance of purified thioredoxins at 280 nm, (·····) pI 4.7 thioredoxin, (——) pI 4.68 thioredoxin.

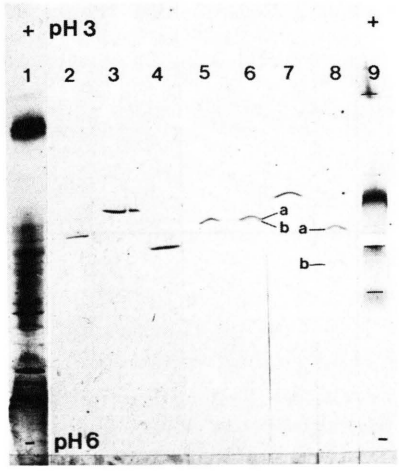

Fig. 6. Determination of pI by IEF for purified thioredoxins. Isoelectric focusing of thioredoxins from 2: *Synechococcus,* app. pI 4.7, 3: *Escherichia coli,* app. pI 4.45, 4: spinach leaf extract, app. pI 5.05, 5: spinach leaf extract, app. pI 4.7 (R_t 16 min from Fig: 5, 6: spinach leaf extract, (a) app. pI 4.68, (R_t 19 min from Fig: 5) contaminated by (b) pI 4.7 thioredoxin, 7: as lane 3, 8: *Saccharomyces cerevisiae,* (a) app. pI 4.8, and (b) app. pI 5.2, lane 1, 9: standards (marked in 9: glucoseoxydase pI 4.15, ferritin pI 4.55, katalase 5.4. IEF on precotes, pH-interval from pH 3–6, prefocus run .25 kVh, total Vh 1.75, 5 µg per thioredoxin, except for lane 3,4 (10 µg). Thioredoxins (a) and (b) from yeast were copurified on MonoQ 5/5, R_t 9 min.

homogeneity as observed in SDS-PAGE; in addition, no differences in their molecular weight was observed (13500 ± 1000, except for *Synechococcus spec.* M_R $15\,500 \pm 1000$, data not shown). The yeast thioredoxin contained two proteins with different pI values (pI 4.8 and 5.2, assumingly identical with thioredoxin II and I [9]) which were copurified. (From work in progress it appears that the PAPS-reductase preferentially uses the more neutral thioredoxin (pI 5.2) by a factor of 30). Only one thioredoxin was observed by this method to occur in the two bacteria investigated (pI 4.45 for *E. coli,* and pI 4.8 for the thermophile *Synechococcus*).

Discussion

The reduction of PAPS in yeast and *E. coli* has previously been observed to require thioredoxin as reductant [9, 10]. The reaction is catalyzed by a PAPS-reductase (also called PAPS-sulphotransferase in the earlier literature [*cf.* 20]) which forms free sulphite and 3′,5′-PAP as reaction products [21, 22]. Although the enzymatic mechanism is not yet fully elucidated, it seems very likely that thioredoxin serves as a electron-transferring cosubstrate in this reaction [*cf.* 21] rather than sulphite-carrier. In the present paper, the possibility was investigated of using the PAPS-reductase as enzymatic probe for thioredoxin. The enzyme system as described above combined a high affinity for thioredoxin (K_M 2 μmol/l for the homologous thioredoxin) with a low specificity concerning the origin of this protein. The high affinity was desirable as it implies a high sensitivity (ranging from 0.1×10^{-9} to 1×10^{-6} M for the homologous thioredoxin). The lack of specificity may be advantageous in the search for thioredoxins from non-related species as well as in the search for multiple forms. *In vitro,* the heterologous systems using thioredoxins from eukaryotic organisms attained at least 50% of the rate of the homologous system. In its low specificity, the PAPS-reductase from *E. coli* resembled the plant NADP malate dehydrogenase, which was activated by thioredoxin *f* and *m* alike [23–25]. The assay, however, appeared less laborious than the spectrophotometric measurement of the NADPH malate dehydrogenase.

By using the PAPS-reductase as probe, a large number of proteins supporting the enzyme as reductant were separated from a spinach leaf extract. Except for a non identified thioredoxin *f,* the PAPS-reductase reported virtually all the thioredoxins which would have been detected by the FbPase- or MDH-assay. The multiplicity of thioredoxins as indicated may be of several reasons. Primarily it seemed to be due to the use of more advanced ion exchangers which have a considerably higher resolution than the conventional celluloses. It was thus possible to separate two thioredoxins with an apparent pI of 4.7 and 4.68 using a strongly acidic anion exchanger (MonoQ 5/5). Secondly, the chloroplast FbPase or MDH may have discriminated between cytosolic and chloroplasmic thioredoxins as compared to the PAPS-reductase. Thirdly, as the major objection, the PAPS-reductase may lack specificity for thioredoxin but also detect disulphides with thioredoxin properties such as glutaredoxin. In deed, PAPS-reductase was observed to use glutaredoxin instead of thioredoxin in the mutant *E. coli* trx A 7004 [10]. In this property, the PAPS-reductase resembles the ribonucleotide reductase which was found to reduce CDP with a 10-fold higher molecular activity using glutaredoxin instead of thioredoxin [26]. Still, Follmann and coworkers [3, 27] demonstrated that the ribonucleotide reductase can be used as probe for thioredoxin *f* or *m* derived from green algae or cyanobacteria. Crossreactivity is, however, not restricted to PAPS-reductase or ribonucleotide reductase: FbPase and MDH as indicator enzymes were also liable to activation by small proteins other than thioredoxin [28, 29]. Consequently, the identification of thioredoxins solely based on their function (either in enzyme activation or in their role as hydrogen donating cosubstrate) can be misleading. At present, elucidation of the primary structure seems indispensable for the identification.

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