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Yeast PAPS reductase: properties and requirements of the purified enzyme

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Abstract. The enzymatic mechanism of sulphite formation in *Saccharomyces cerevisiae* was investigated using a purified 3′-phosphoadenylsulphate (PAPS) reductase and thioredoxin. The functionally active protein (M_R 80–85 k) is represented by a dimer which reduces 3′-phosphoadenylyl sulphate to adenosine-3′,5′-bisphosphate and free sulphite at a stoichiometry of 1:1. Reduced thioredoxin is required as cosubstrate. Examination of the reaction products showed that free anionic sulphite is formed with no evidence for "bound-sulphite(s)" as intermediate. V_{max} of the enriched enzyme was 4–7 nmol sulphite · min^{-1} · mg^{-1} using the homologous thioredoxin from yeast. The velocity of reaction decreased to 0.4 nmol sulphite · min^{-1} · mg^{-1} when heterologous thioredoxin (from *Escherichia coli*) was used instead. The K_m of homologous thioredoxin was $0.6 \cdot 10^{-6}$ M, for the heterologous cosubstrate it increased to $1.4 \cdot 10^{-6}$ M. The affinity for PAPS remained practically unaffected ($K_{m\ PAPS}$: $19 \cdot 10^{-6}$ M in the homologous, and $21 \cdot 10^{-6}$ M in the heterologous system). From the kinetic data it is concluded that the enzyme followed an ordered mechanism with thioredoxin as first substrate followed by PAPS as the second. Parallel lines in the reciprocal and a common intersect in the Hanes-plots for thioredoxin were seen as indication of a ping-pong (with respect to thioredoxin) uni-bi (with respect to PAPS) mechanism.

Key words: 3′-Phosphoadenylyl sulphate reductase – Sulphite formation – Cysteine biosynthesis – Thioredoxin – *Saccharomyces cerevisiae* – HPLC enzyme analysis

Inorganic sulphate is assimilated by a sequence of enzymatic steps which convert hexavalent sulphate to divalent organic sulphur as required for cysteine biosynthesis. Yeasts like enterobacteria use PAPS (3′-phosphoadenylylsulphate) as substrate for the formation of sulphite sulphur as intermediate. This reduction is catalyzed by a PAPS reductase. First evidence for this enzyme was obtained by Wilson et al. (1961). The complete PAPS reducing system in yeast consisted of three proteins, two of which were later identified as a thioredoxin reductase ("fraction A", EC 1.6.4.5) and thioredoxin ("fraction C"). The PAPS-reductase ("fraction B", not classified by EC) was not well characterized or studied as a purified enzyme. The activity is measured as the sulphite formation from PAPS (Bandurski 1971). It remained uncertain whether free inorganic sulphite or an enzyme- or carrier bound sulphite was formed as the product of this reaction. Wilson and Bierer (1976) renamed the enzyme "sulpho-transferase" because they could find confirmatory evidence for a transfer of the sulphonyl group from PAPS onto an unidentified carrier as originally proposed by Torii and Bandurski (1967):

$$PAPS^{4-} + \text{carrier-}S^{-} \rightarrow \text{carrier-}S{:}SO_3^{-} + PAP^{4-}.$$

In its formation of a "carrier-bound sulphite" as product rather than free sulphite the yeast enzyme resembled a bound-sulphite forming APS sulphotransferase which was described for algae and plants (Abrams and Schiff 1973; Schmidt 1973; review in Schiff and Fankhauser 1981). This proposal was based on the occurrence of exchangeable sulphite upon gel filtration or paper electrophoresis of a complete reaction mixture containing enriched PAPS reductase activity:

$$\text{carrier-}S{:}{}^{35}SO_3^{-} + SO_3^{2-} \longleftrightarrow \text{carrier-}S{:}SO_3^{-} + {}^{35}SO_3^{2-}.$$

As yeast was observed to contain a thioredoxin requiring sulphate reducing system (Porqué et al. 1970) it has been speculated that thioredoxin is identical with the sulphite carrier, hence, S-sulpho-thioredoxin represented the bound sulphite.

$$\text{Thio}\begin{matrix}\diagup \text{SH} \\ \diagdown \text{SH}\end{matrix} + \text{PAPS} \rightarrow \left[\text{Thio}\begin{matrix}\diagup \text{SH} \\ \diagdown \text{S:}SO_3^{-}\end{matrix}\right] + \text{PAP} + H^{+}.$$

Until today, the hypothetical carrier in yeast or algae has not been identified. The only PAPS-reducing enzyme which was investigated more thoroughly was isolated from *Escherichia coli* (Tsang 1981, 1983; Schwenn and Schriek 1987). It was observed to form free sulphite with thioredoxin as hydrogen donor. No evidence for S-sulpho-thioredoxin as intermediate was obtained. Formation of protein-bound sulphite by this enzyme was observed as artifact (Schriek 1985). In view of the controversy about the true nature of the reaction product and mechanism of a PAPS-reductase

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Abbreviations: APS, adenylyl sulphate; DTE, dithioerythritol; DTT, dithiothreitol; ; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LSC, liquid scintillation counting; 3′,5′-PAP, adenosine-3′,5′-bisphosphate; PAPS, 3′-phosphoadenylyl sulphate; PEP, phospho-(enol)pyruvate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol

we purified the enzyme and the cosubstrate extensively with the aim to identify these products and to investigate the stoichiometry, kinetics and enzyme constants of the PAPS-reductase from *Saccharomyces cerevisiae*.

Materials and methods

Isolation and assay of PAPS-reductase and thioredoxin

Commercial baker's yeast was used for the isolation of enzyme and thioredoxin.

1. PAPS-reductase. (a) Crude extract. – 500 g yeast were treated with toluene/bicarbonate according to Shapiro (1971), but supplemented with EDTA (1.3 g) and β-mercaptoethanol (17.2 mmol). After centrifugation ($20,000 \times g$, 30 min) 740 ml autolysate were obtained containing 7,814 mg protein.

(b) pH-treatment. To the crude extract acetic acid (1 M) was pipetted until the pH reached 6.2 (at 8 – 10° C). After centrifugation as before a bright yellowish extract was obtained (850 ml, 6,732 mg protein).

(c) Ammonium sulphate fractionation. The pH of the extract was adjusted to 7.6 with Tris (free base, 2 M) before 315 g of ammonium sulphate were added in small portions. The protein was allowed to settle overnight. The supernatant was decanted leaving 435 ml of a protein suspension. Further steps were carried out using 1/5 of this precipitate. The protein was collected by centrifugation as before and dissolved in a final volume of 50 ml in Tris-HCl 50 mM (pH 8.0) including EDTA 1 mM and DTT 5 mM (57.5% sat precipitate: 502.5 mg protein per 50 ml).

(d) Hydrophobic chromatography. Ammonium sulphate was added to the protein solution to give a final concentration of 1 M (155 mS cm^{-1}). The solution was allowed to rest for 30 min at 0° C before sedimenting insoluble proteins by centrifugation ($27,000 \times g$, 30 min). The supernatant (54 ml, 393 mg protein) was collected on a column (10.5×1.6 cm) packed with Phenyl Sepharose CL 4B (Pharmacia/LKB, Freiburg, FRG) equilibrated with Tris-HCl 50 mM (pH 8.0) in ammonium sulphate 1 M and EDTA 1 mM. The enzyme was eluted by applying a descending gradient of ammonium sulphate from 1 to 0 molar buffered in Tris-HCl/EDTA as for equilibration. The enzyme emerged at appr. 0.17 molar of ammonium sulphate; the most active fractions were combined: 97 ml, 121 mg protein.

(e) Gelfiltration. The protein from the previous step was precipitated with ammonium sulphate (43.6 g, 65% sat), centrifuged as before and resuspended in a minimum volume of Tris-HCl/EDTA buffer, supplemented with DTE 5 mM. It was applied to a column (48×2.2 cm) of Sephacryl S200 (Pharmacia/LKB, Freiburg, FRG), equilibrated with the same buffer. The enzyme emerged at a volume corresponding to the elution volume of bovine serum albumin (M_R 68 k), 19 ml, 44.7 mg protein.

(f) Ion exchange chromatography. Brief dialysis against Tris-HCl/EDTA/DTE buffer was required to enable the collection of protein on a DEAE-Trisacryl column (8×105 mm). The column was developped with a linear gradient of NaCl (0 – 350 mM in Tris-HCl 50 mM, EDTA 0.1 mM and DTE 1 mM, total volume 250 ml) at a flow rate of 0.3 ml/min. The enzyme emerged at a concentration of 140 to 170 mM of Cl^-. The most active fractions were combined: 11.5 mg protein in 13.5 ml.

(g) Concentration. The fractions were concentrated by adsorption onto a small column of Phenyl Sepharose (8×65 mm) under conditions as outlined in step (d). Desorption was achieved in a single step using the Tris/EDTA/DTE-buffer as eluent (1.9 mg protein in 2.6 ml).

2. PAPS-reductase: determination of enzymatic activity. The enzyme was measured as thioredoxin-dependent formation of $^{35}SO_3^{2-}$ from [^{35}S]-PAPS with dithiothreitol (DTT, or dithioerythritol, DTE) as auxiliary reductant for thioredoxin. The assay included Tris-HCl 50 mM (pH 8.0), EDTA 1 mM, NaF 25 mM, DTT (or DTE) 5 mM, thioredoxin 4.5 µg protein (from *Escherichia coli*) or 1.4 µg (from *Saccharomyces cerevisiae*), Na_2SO_3 40 mM as carrier sulphite, [^{35}S]-PAPS 5 – 25 µM (specific radioactivity 414×10^3 to 88×10^3 Bq $nmol^{-1}$) and enzyme in a total volume of 100 µl. Thioredoxin from *E. coli* was used instead of the homologous protein when column effluents were monitored for PAPS-reductase activity. This thioredoxin was isolated from an overproducing strain of *E. coli* K12 containing a plasmid encoded trxA gene (SK 3981) (Lunn et al. 1984). One unit was defined as 1 µmol sulphite min^{-1} formed from PAPS using the yeast thioredoxin.

3. Thioredoxin: isolation. The pH-treated extract was precipitated with ammonium sulphate (75% sat), resuspended in Tris-HCl 50 mM (pH 8.0), EDTA 1 mM and dialyzed against the Tris-buffer reduced 1/10 in strength. The protein was then heated to 70° C for 5 min, centrifuged ($28,000 \times g$, 30 min) and adjusted to a conductivity of 1.2 mS cm^{-1} with distilled water before separation on a DEAE-TSK 650 S gel (Merck, Darmstadt, FRG), Trisacryl DEAE, PBE94 and MonoQ 5/5 (Pharmacia/LKB, Freiburg, FRG) as described recently (Schwenn and Schriek 1987). The thioredoxin isolated by this method, very likely represented the "thioredoxin II", originally described by Porqué et al. (1970).

4. Thioredoxin: assay and identification. Yeast thioredoxin was assayed as cosubstrate in the reduction of PAPS to sulphite catalyzed by the PAPS-reductase from *E. coli* and yeast. The validity of this assay was previously confirmed by comparison with thioredoxin-dependent enzyme activation [NADP:malate dehydrogenase for m-type thioredoxins, and fructose-1,6-bisphosphatase for f-type thioredoxins (Schwenn and Schriek 1987)]. Additional parameters for the identification were cross-reaction with polyclonal monospecific antibodies against *E. coli* thioredoxin and determination of the retention on a calibrated HPLC size exclusion gel (Zorbax GF250, NEN duPont, Bad Nauheim, FRG) using authentic thioredoxin from *E. coli* as a reference.

Other methods

1. Reversed-phase paired-ion HPLC. The nucleotides, sulphate and sulphite were separated by the HPLC method as described earlier (Schwenn and Jender 1980). In addition to the UV-integrator (Spectra-Physics, Darmstadt, FRG) an integrating HPLC radioactivity monitor (IM2000, IsoMess, Straubenhardt, FRG) was used for the determination of ^{35}S labelled compounds (Jender and Schwenn 1984).

2. Ion exchange HPLC. A high performance column (8×85 mm, containing DEAE TSK 650S) was used for the

Table 1. Purification of yeast PAPS reductase

Step	Volume (ml)	Concentration (mU/ml)	Amount (mU)	Protein (mg/ml)	Spec. activity (mU/mg)	Yield [%]	Purification
a: crude extract	740	3.58	2,645	10.6	0.4	100	1
b: pH-treatment	850	3.06	2,610	7.9	0.4	98	1.1
c: precipitation	250	14.58	3,645	10.0	1.4	137	4.2
d: phenyl-sepharose	485	9.45	4,583	1.2	7.5	173	22.2
e: sephacryl	95	20.45	1,942	2.3	8.7	73	25.8
f: DEAE-trisacryl	67.5	15.04	1,015	0.85	17.52	38	51.8
g: phenyl-sepharose	13	32.1	417	0.73	44.13	16	130.6

Procedure for the purification of PAPS-reductase using homologous thioredoxin (1.4 µg) under assay conditions as outlined in Materials and methods. Due to endogeneous thioredoxin still present during the first steps, the activity as obtained for steps a–c may be unreliable; steps d to f were carried out repetitively using only 1/5 of the starting material. The values were then extrapolated for the total protein deriving from 500 g of yeast

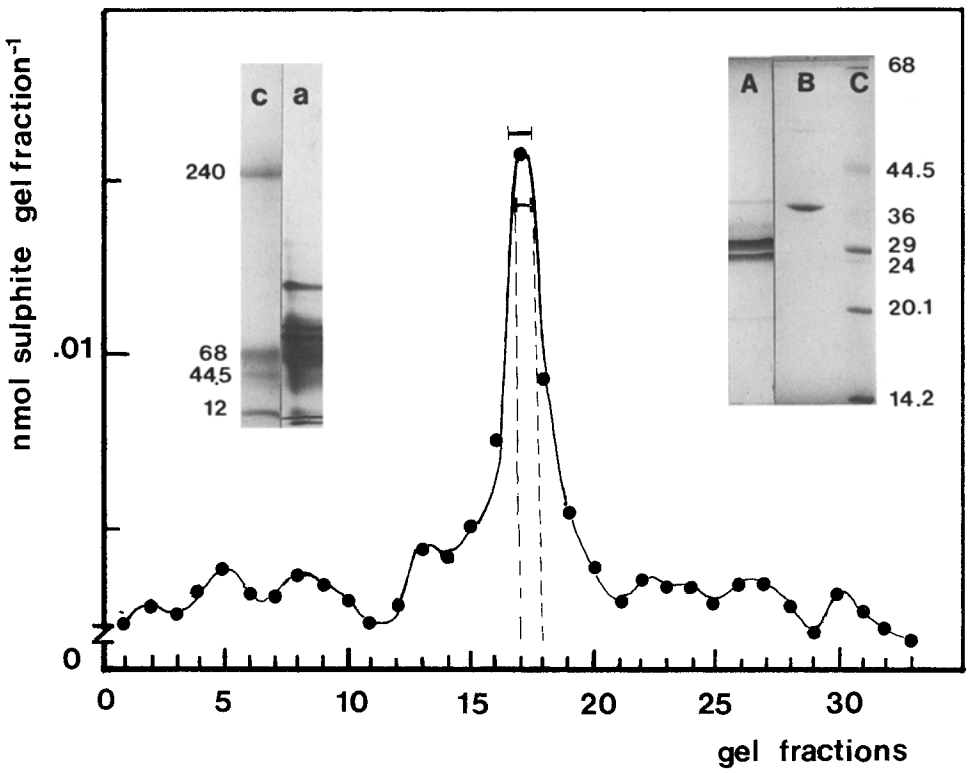

Fig. 1
PAPS reductase from *Saccharomyces cerevisiae* as analyzed by gel electrophoresis. Localization of the enzyme activity [native system, 7.5% cross-linked (Davis 1964)] by incubating gel slices (3 mm) with PAPS and thioredoxin as described for the complete enzyme assay mixture (Materials and methods, p 314), and analyzing for sulphite formation. Protein used for SDS gel electrophoresis is indicated by horizontal bar |—|. Insets, *lane a*: PAPS-reductase from step "g", 100 µl; *lane c*: weight marker proteins, $M_R \times 10^3$, native disc system – *A*: PAPS-reductase as before, 25 µl, *B*: re-electrophoresed protein from native disc system, *C*: weight marker proteins, $M_R \times 10^3$, as separated by SDS-PAGE (15% cross-linked)

separation by applying a linear gradient of NaCl from 0 to 400 mM in Tris-HCl 50 mM (pH 8.0) and EDTA 0.1 mM. The gradient was run within 180 min at a flow rate of 0.5 ml min^{-1} (Waters Assoc., Modell 660 solvent programmer in combination with two Modell 6000A HPLC pumps). Identification of the compounds was by chromatography of authentic samples, but sulphite which was also localized by distillation of SO_2.

3. Size exclusion gel filtration. The isolated enzyme protein, thioredoxin or complete enzyme reaction mixtures were separated on a calibrated gel filtration column (Zorbax GF250 and GF450) using KH_2/K_2HPO_4 buffer at 50 mM (pH 6.8) and Na_2SO_4 200 mM as mobile phase at a flow rate of 0.5 ml min^{-1}. Fractions of 250 µl were collected and assayed for the individual enzyme or cofactor activity or for products formed by the complete enzyme system.

4. Isoelectric focusing and gel electrophoresis. Servalyt precotes (Serva, Heidelberg, FRG) with a pH range of 3–10 were used for the analysis of complete enzyme reaction mixtures. Samples of 20 µl were applicated to the precote, prefocused at 400 V until a current of 1 mA was reached. The focusing run was for 60 min at 1,700 V which was attained by stepwise increasing the voltage (200 V/20 min), when the power level was preset to 4 VA. Focusing was completed when a constant current of 1 mA was read over 15 min. Fixation and staining of the precotes was carried out as described in the manufacturer's instructions using tri-chloroacetic acid and Coomassie Brilliant Blue R250. Radioactivity was spotted by exposure (6 months) of the dried precotes to Kodak X-Omat AR diagnostic film. Strips were cut out from the precotes and X-ray film and scanned with an ISCO 1310 gel scanner at 580 nm for proteins and for radioactivity as indicated for labelled compounds by

staining of the X-ray film. The molecular weight of the subunit of PAPS reductase was determined on SDS-poly acrylamide gels (15% crosslinked) whereas 7.5% crosslinked gels were used for non-denaturing gel electrophoresis. The enzyme was localized in these gels by incubating the gel slices without further treatment in 100 µl of a reaction mixture as described above for determination of the enzyme activity.

5. Radioactive compounds, determination of protein, and statistical methods. Labelled PAPS was prepared from [^{35}S]H_2SO_4 (carrier free), ATP (regenerated by PEP and pyruvate kinase), ATP sulphurylase (Sigma, Taufkirchen, FRG), pyrophosphatase (Boehringer, Mannheim, FRG) and APS kinase purified from yeast (Schriek and Schwenn 1986). ^{35}S-labelled compounds were measured with LSC cocktails for aqueous samples in a LKB 1219 β-counter equipped with a fraction plot option. Internal standardization was used for the appropriate correction of chemoluminescence. ^{35}S-sulphite was determined as acid-volatile SO_2 employing tri-octylamine for absorption (Schriek and Schwenn 1986). Coomassie dye binding was used for the determination of protein (Bradford 1976). Curve fitting and statistical analysis was done with software from Microware (State College, PA, USA).

Results

Enzyme and thioredoxin

The PAPS reductase was purified by a combination of hydrophobic interaction chromatography gel filtration and ion exchange chromatography. The procedure as outlined in Table 1 removed the endogeneous thioredoxin efficiently enabling the investigation of its role in the reduction of PAPS. The enzyme was not homogeneous (Fig. 1) after these six steps, but pure enough to be used for the determination of enzyme constants. A homogeneous protein was obtained after two dimensional gel electrophoresis [discontinuous according to Davis (1964) as first dimension followed by SDS-PAGE according to Laemmli (1970)]. The protein band detected by enzyme activity (lane a, Fig. 1) was re-electrophoresed in the SDS gel system (lane B, Fig. 1) showing a molecular weight of 36 k per subunit. As non-denaturing electrophoresis gave a molecular weight of approximately 80 to 85 k, and HPLC size exclusion showed a mass of 66 – 68 k the active enzyme assumingly represented a dimer. HPLC analysis (and disc electrophoresis) of the complete reaction mixture gave no evidence of a stable complex formed by the enzyme and thioredoxin or PAPS.

The minimal requirements for PAPS-reduction by the enzyme were the two substrates thioredoxin and PAPS (Table 2). For continuous reduction an auxiliary reductant was necessary; DL-1,4 dithiothreitol (DTT) or NADPH (including the NADPH:thioredoxin oxidoreductase) were tested. DTT (and also DTE) reduces thioredoxin in a purely chemical fashion (Luthman and Holmgren 1982) whereas NADPH in non-phototrophic organisms is the physiological reductant for thioredoxin (Holmgren 1985). As thioredoxin was isolated in a partly reduced state, residual rates of sulphite formation were observed in the absence of reductants already (Table 2). The concentration of DTT as used in the tests was exceeding the saturating concentration by a factor of 10^3. The homologous thioredoxin could be replaced by heterologous thioredoxin (from *Escherichia coli*); the rate of enzyme (v_{app}) was lowered in this case to 1/3 of the homologous system (Fig. 2). Linearity of the assay in the homologous system was maintained for 30 min using 50 µM PAPS. A reduction of PAPS using the physiological reducing system with a NADPH:thioredoxin reductase from *E. coli* did, however, not work with the yeast thioredoxin and enzyme. The NADPH dependent reduction was observed to operate sufficiently only with the bacterial PAPS reductase and thioredoxin, presumably because of the known specificity of the thioredoxin oxidoreductase for its homologous thioredoxin (Holmgren 1985).

Table 2. Requirements of the yeast PAPS-reductase

	dpm	nmol SO_3
1. DL-dithiothreitol as reductant:		
Enzyme, thioredoxin (yeast)[a]		
DDT	101,509	0.67
– thioredoxin	639	0
– enzyme	234	0
– DTT[c]	72,075 – 3,401	0.48 – 0.02
2. NADPH as reductant:		
Enzyme, thioredoxin (yeast)[b]		
NADPH, thioredoxin-reductase (*Escherichia coli*)	12,250	0.08
– enzyme	149	0
– thioredoxin, – enzyme	118	0
– thioredoxin-reductase, NADPH	40,591	0.27
– NADPH	17,800	0.12
Complete, but thioredoxin from *E. coli*	41,904	0.28
PAPS-reductase and thioredoxin from *E. coli*	298,078	1.96

Enzyme from step g, 5 µl, was incubated in the standard assay mixture for 5 min and analyzed for sulphite formed (carrier sulphite included for distillation). NADPH:thioredoxin oxidoreductase (12 µg) and PAPS-reductase (8 µg) were partially purified from *E. coli*, the bacterial thioredoxin was homogeneous (2.3 µg)
[a] 1.4 µg and [b]0.7 µg of homologous thioredoxin, [c]residual reduction in the absence of DTT by thioredoxin was due to the reduced form of the protein which disappeared when kept unprotected from oxidation. Controls using DDT as reductant for the NADPH-dependent assays of yeast PAPS-reductase were: 0.3 nmol (0.7 µg yeast thioredoxin), 0.28 nmol (1.1 µg bacterial thioredoxin); the enzyme/thioredoxin system from *E. coli* gave rise to 2.25 nmol sulphite under the same conditions

Reaction products and stoichiometry of reaction

A current hypothesis for the role of thioredoxin suggested that an intermediate between PAPS and the product sulphite (e.g. S-sulpho-thioredoxin) was formed (Schiff and Fankhauser 1981; Trudinger and Loughlin 1981). Analysis by HPLC size exclusion chromatography of the complete reaction mixture (containing the purified enzyme, thioredoxin and PAPS) gave no indication of S-sulpho-thioredoxin formation, irrespective of the presence of DTT as auxiliary reductant (Fig. 3). Free inorganic sulphite was found exclusively, the omittance of DTT only resulted in a low to immeasurable formation of sulphite from PAPS. Free sulphite was also observed when a complete reaction mixture was separated by HPLC ion exchange chromatography

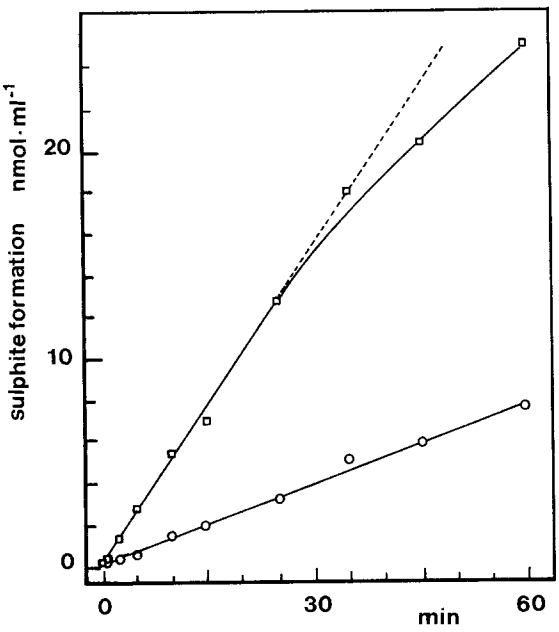

Fig. 2. Progress curves for the enzyme from *S. cerevisiae* using homologous and heterologous thioredoxins. Apparent velocities as determined by distillation of acid volatile sulphite formed from [^{35}S]PAPS in the presence of 3.6 µg partially purified enzyme, yeast thioredoxin (1.4 µg) or thioredoxin from *Escherichia coli* (4.5 µg), other reaction conditions as described in Materials and methods, but volume which was increased as to enable the analysis of 100 µl samples at intervals as indicated. □: yeast thioredoxin, ○: *E. coli* thioredoxin

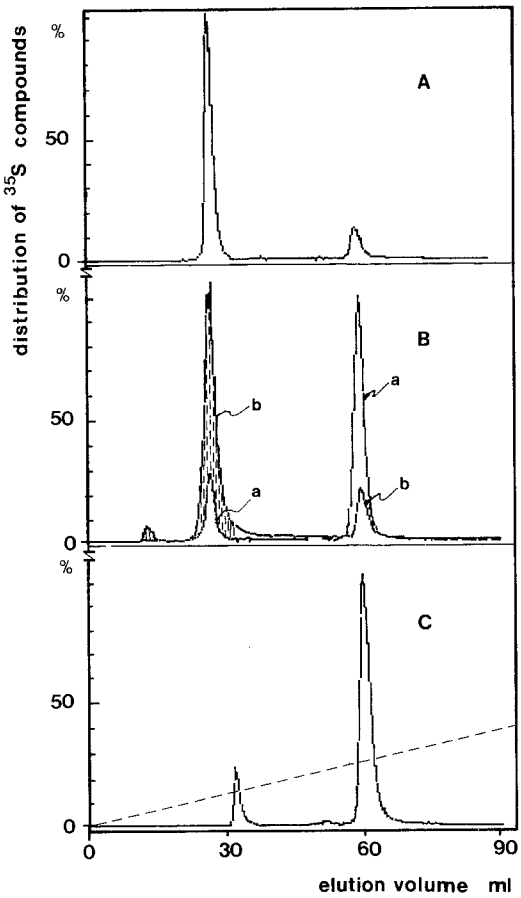

Fig. 3 A – C. Identification of the reaction products I: Ion exchange chromatography. **A** complete reaction mixture of PAPS-reductase including unlabelled carrier sulphite (20 mM) incubated for 10 min. **B** trace (*a*) as before, but carrier sulphite omitted, trace (*b*) as reaction conditions as in (*a*) but sample incubated for 60 min, **C** control, no enzyme reaction permitted. Detection of radioactivity by on-line scintillation counting as eluted from a DEAE TSK 650 S column developped with a linear gradient of NaCl (dashed line in **C**, 2 mM Cl^-/ml). Identification and quantitation of ^{35}S-compounds: **A** sulphite (V_{eff} 23.2 ml) 4.0 nmol, PAPS (V_{eff} 60.5 ml) 0.7 nmol; **B** trace (*a*) sulphite (V_{eff} 26.6 ml) 0.8 nmol, PAPS (V_{eff} 61.5 ml) 3.2 nmol, APS (V_{eff} 57 ml) 0.1 nmol – trace (*b*) sulphite (V_{eff} 27.5 ml) 3.5 nmol, PAPS (V_{eff} 61.8 ml) 0.6 nmol, APS (V_{eff} 52 ml) 0.1 nmol, unidentified exchangeable sulphite (V_{eff} 12.8 ml) 0.15 nmol. **C** sulphate (V_{eff} 33.5 ml) 0.6 nmol, PAPS (V_{eff} 63 ml) 4.7 nmol, APS (V_{eff} 52.5 ml) 0.07 nmol. Determination by distillation of acid volatile SO_2 of individual compounds obtained from **B** trace (*b*) (indicated as hatched areas): unidentified exchangeable sulphite: 0.09 nmol free anionic sulphite 0.62 nmol

(Fig. 4, trace A). In the case of extended incubation periods (Fig. 4, trace B) when carrier sulphite and additional DTT were omitted a small amount of exchangeable sulphite (3.2%) was revovered from an unidentified compound which by its elution behaviour was not free sulphite. As observed during HPLC gel filtration, this compound was formed from sulphite and DTT already in the absence of enzyme.

Previous reports of a "bound sulphite" as intermediate of the PAPS reduction in bacteria or yeasts made use of electrophoretic separations [paper electrophoresis (Torii and Bandurski 1967; Tsang and Schiff 1976a; Wilson and Bierer 1976)]. Isoelectric focusing of complete reaction mixtures using the purified enzyme and thioredoxin confirmed the observation, that free sulphite was formed by the PAPS-reductase. But, in comparison to the two chromatographic methods, a non-specific labelling of proteins still present in the reaction mixture was observed. The lack of specificity is enlightened by the finding that bovine serum albumin could be labelled with sulphite when added to the enzyme assay (data omitted).

As carrier sulphite seemed indispensable for a complete recovery of sulphite formed from PAPS, kinetics and product stoichiometry were measured with carrier supplemented samples. In this kinetic investigation, samples were withdrawn from a reaction mixture at 5 min intervals and analysed for PAPS, 3′,5′-PAP, SO_3^{2-} and its oxidation product SO_4^{2-} by reversed phase paired-ion HPLC. The progress curve (Fig. 5) showed that PAPS was reduced to sulphite and 3′,5′-PAP at a molar ratio of 1:1. The kinetics of reaction was linear with no indication of an intermediate between PAPS and sulphite; the rates of 3′,5′-PAP and sulphite formation were parallel and identical.

An optimized reaction system (pH 8.0–8.2, DTT $\geq$ 0.2 mM) was used for the determination of affinity constants. The K_m values were obtained from slope replots of the corresponding Hanes-plots (data omitted) of S versus Sv^{-1}. Assuming homogeneous thioredoxin with a molecular weight of 12 k the $K_{m\,Thio}$ was 0.6 µM and for PAPS $K_{m\,PAPS}$ 19 µM. V_{max} from these data was 4–7 nmol sulphite min^{-1} mg^{-1}. Replacing the homologous thioredoxin with the *E. coli* thioredoxin as alternative substrate gave a lowered affinity (K_m 1.4 µM) for this substrate without affecting the affinity for PAPS considerably (K_m 21 µM). V_{max} in the

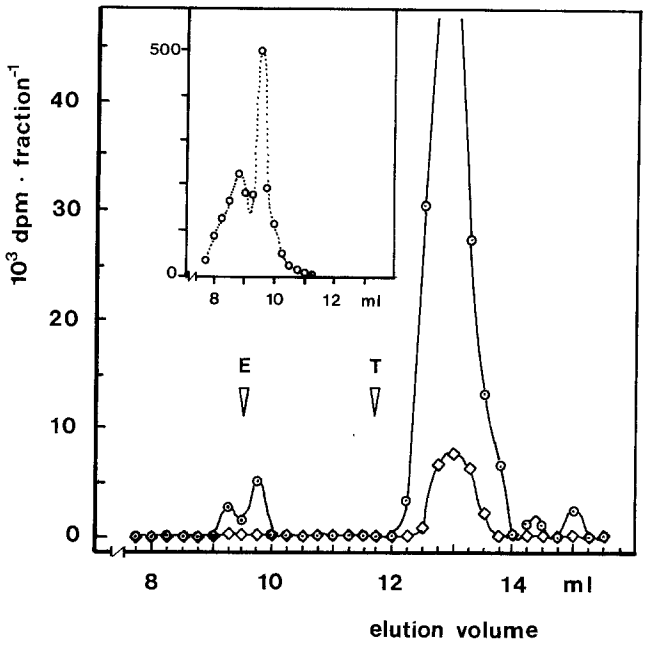

Fig. 4. Identification of reaction products II: HPLC size exclusion. Separation of a PAPS-reductase assay mixture on an HPLC gel filtration column, carrier sulphite omitted. The enzyme was allowed to react for 10 min with its substrates before separation and collection of 250 µl fractions. Enzymatic activity was localized by the assay as outlined in Materials and methods using 25 µl of the fractions (Inset), the sulphite was determined as acid-volatile SO_2 distillable from each of the fractions after supplementation with 40 µmol unlabelled carrier sulphite. ⊙: total radioactivity, ◇: distillable sulphite; Inset: ○: sulphite formed by PAPS-reductase after HPLC separation. *E*: enzyme, *T*: thioredoxin

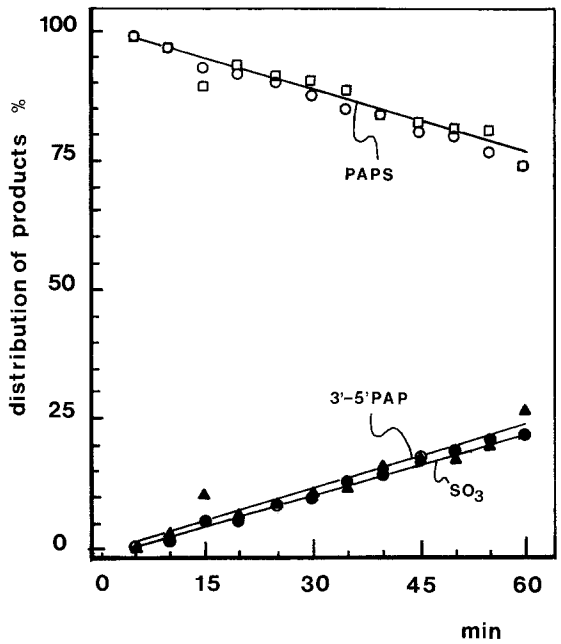

Fig. 5. Stoichiometry of the reduction of PAPS to sulphite and 3′,5′-PAP. Progress curve of appearance of products and disappearance of substrate as analyzed by reversed-phase paired-ion HPLC. Samples were withdrawn from a PAPS-reductase reaction mixture (homologous system, but otherwise identical to sample described in the legend to Fig. 2) at intervals as indicated and analyzed for reaction products by UV-absorption and radioactivity. 100% full scale equals 5 nmol. ^{35}S-PAPS: ○; $^{35}S-SO_3$: ●; PAPS (254 nm): □; PAP (254 nm): ▲

heterologous assay, as anticipated by the data in Fig. 2, dropped drastically to 0.4 nmol sulphite min^{-1} mg^{-1}.

Discussion

PAPS-reduction in *Saccharomyces cerevisiae* was catalyzed by an enzyme which used thioredoxin and PAPS as substrates and formed free anionic sulphite and 3′,5′-PAP as products. A requirement for other cofactors was not observed. A bound sulphite as described in previous investigations was not found and no evidence for a formation of S-sulpho-thioredoxin as intermediate has been obtained. Kinetic investigations of the enzyme reaction supported the view that bound sulphite followed rather than preceded the formation of sulphite. Side reactions in which the sulphite reacted with proteins or oxidized dithiols non-specifically seem to explain the occurrence of bound sulphites. The diversity of side products in the presence of thiols and dithiols has been described by Tsang and Schiff (1976b) for the analogous APS sulphotransferase from *Chlorella* spec. The authors found that mono-thiols like glutathione formed S-sulpho-compounds like $GS:SO_3H$ predominantly whereas dithiols like DTT or DTE gave rise to free anionic sulphite due to an intramolecular reduction. In view of the redox potentials envolved a separation into S-sulpho-compounds forming and free sulphite forming thiols appears unjustified. The redox potential for the $S:S/(SH)_2$ couple is in the range of $\Delta E^\circ - 260$ mV (Jocelyn 1972), for the $S:SO_3^{2-}/SH^-SO_3H^-$ couple it is $\Delta E^\circ - 402$ mV (Thauer et al. 1977). As ΔE of both reactions is 142 mV, disulphides are suitable oxidants for sulphite leading to the formation of thiosulphates. The formation of sulphite, therefore requires excess of thiol (10^4 to 10^5) over disulphides, or disulphides should be absent from the enzyme reaction mixture. With crude enzyme preparations this may be impossible to achieve so that bound sulphites were formed as soon sulphite was produced by the enzyme. With a considerably purified enzyme (and thioredoxin) formation of a specific "bound sulphite" was not observed. In addition, S-sulpho-thioredoxin as intermediate of the reduction was not found under conditions suitable for its detection (HPLC gel filtration). As discussed below, the possible reaction mechanism (ping-pong with respect to thioredoxin and uni-bi with respect to PAPS) is inconsistent with S-sulpho-thioredoxin. The catalytic activity of the PAPS reductase was dependent on thioredoxin. The affinity for this substrate was very high ($K_m \approx 1$ µM). The common intersect on the S/v-axis in the Hanes-plot may be seen as indication of a ping-pong substrate-enzyme reaction. A ping-pong mechanism seems to be characteristic of thioredoxin dependent enzymes (Holmgren 1985). If the enzyme truely follows such a mechanism, the formation of S-sulpho-thioredoxin as intermediate would have to be excluded. In fact, Tsang (1983) reported that the enzyme from *Escherichia coli* could react with PAPS and form sulphite after reduction with and separation from the thioredoxin. This order of reaction coincides with a ping-pong mechanism. PAPS seemed to be metabolized with an affinity independent of the thioredoxin used because in the heterologous assay V_{max} was lowered without considerable effect on the K_m PAPS (19 µM in the homologous and 21 µM in the heterologous assay with *E. coli* thioredoxin).

In view of the data currently available a mechanism is proposed in which the enzyme is reduced first by thioredoxin and secondly, PAPS reacts with the reduced enzyme in a uni-bi mechanism releasing sulphite and 3′,5′-PAP:

$$E_{ox} \xrightarrow[\text{Thio}_{red}\ \downarrow \quad \text{Thio}_{ox}\ \uparrow]{} E_{red} \xrightarrow[\text{PAPS}\ \downarrow \quad \text{PAP}\ \uparrow \quad SO_3^{2-}\ \uparrow]{} E_{ox}.$$

As yet, the order of product release is unknown. An inhibition by sulphite was not observed (at concentrations of 20–40 mM). 3′,5′-PAP was inhibitory but its mechanism was not investigated like for the enzyme from *E. coli* where the product reacted as mixed type inhibitor (Schwenn and Schriek 1987).

Further work using the physiological reducing system NADPH:thioredoxin oxidoreductase and full exploration of the inhibition pattern by products and alternative substrates becomes necessary in order to describe the reaction mechanism in more detail.

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