Sulphate Assimilation in Higher Plants: A Thioredoxin-Dependent PAPS-Reductase from Spinach Leaves

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Higher plant leaf protein was investigated for the enzyme activity catalyzing a thioredoxin-dependent reduction of 3'-phosphoadenylylsulphate (PAPS) to sulphite. The enzyme became detectable when heterologous thioredoxin from *Escherichia coli* was used substituting for the hitherto unidentified plant thioredoxin. The enzyme's cross-reactivity with heterologous thioredoxin enabled the partial purification and brief characterization. The molecular weight of the enzyme as estimated by HPLC size exclusion and gel filtration was 68–72 k. The protein reduced PAPS only when thioredoxin was present as cosubstrate. The function of this enzyme in the assimilation of inorganic sulphate by higher plants is discussed in comparison to the function of the respective enzymes from *Escherichia coli* and *Saccharomyces cerevisiae*.

Introduction

Higher plants meet their requirement for sulphur by the reduction of inorganic sulphate. With the exception of enterobacteria, the pathway of assimilatory sulphate reduction in higher organisms still is a matter of controversy. The crucial question is whether the higher S-autotrophic, and predominantly photo-autotrophic organisms form free inorganic sulphite or a "protein bound" thiosulphonate as intermediate. Thiosulphonate formation was reported to occur in plants, algae and different species of phototrophic bacteria [1]. A sulphotransferase reaction was found responsible for the formation of thiosulphonate from adenylylsulphate (APS) in the presence of a not yet identified thiol [2–4]. A similar mechanism of thiosulphonate formation was proposed for yeast by Wilson and Bierer [5] in which the 3'-phosphorylated adenylylsulphate (PAPS) was used as source of the sulphonate group by a PAPS-sulphotransferase. However, more recent *in vitro* studies of the corresponding enzyme (termed

"PAPS-reductase") showed that primarily free anionic sulphite was formed which then by side reactions gave rise to a "bound sulphite". Investigations of the reaction mechanism indicated that thioredoxin is a ping-pong substrate of the enzyme reacting first. The reduced enzyme then catalyses an uni-bi reaction which converts PAPS into sulphite and adenosine-3',5'-bisphosphate (3',5'-PAP):

$$PAPS + Thio(SH)_2 \rightarrow SO_3H^- + 3',5'\text{-PAP} + H^+ + Thio(S)_2 \qquad (1)$$
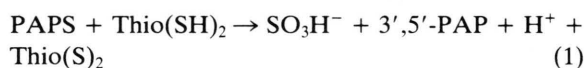
with no evidence for S:sulpho-thioredoxin as intermediate [6]. The reaction mechanism is strikingly similar to the PAPS-reductase of *Escherichia coli* [7, 8].

As plants contain a ferredoxin-dependent sulphite-reductase [9, 10], a thioredoxin-activated APS-kinase [11] and thioredoxins [12, 13], the complete set of enzymes and cofactors is present which would enable the plant to reduce sulphate by a mechanism completely homologous to yeast and bacteria provided that the PAPS-reductase is also active.

The major aim of the present work was to demonstrate the occurrence of a thioredoxin-dependent PAPS-reductase in plants as the "missing link" between APS-kinase and sulphite-reductase. Since higher plants contain at least five different thioredoxins (f_A and f_B, m_b, m_c, and m_d) the problem which of the thioredoxins is used by the PAPS-reductase was eluded by replacing the plant thioredoxin with recombinant thioredoxin (*E. coli*). It was thus possible

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to partly purify and characterize the enzyme from spinach leaves.

Materials and Methods

Purification and enzymatic assay of PAPS-reductase

The enzyme was extracted from spinach leaves using phosphate buffer 50 mM (pH 7.7), EDTA 1 mM, PMSF 1 mM, 10 mM β-MSH, and 5% (g/v) of polyvinylpyrrolidone ("Polyclar AT") at a ratio of 1000 ml/kg of deribbed leaves. Cell debris were removed by filtration and centrifugation ($10,000 \times g$, 30 min), insoluble particles were precipitated with ammonium sulphate (35% saturation at 4 °C) followed by a centrifugation as before. Crude leaf protein was collected from the precipitate forming between 35 and 80% saturation with ammonium sulphate. This fraction was used likewise for the preparation of enzyme or thioredoxin.

The PAPS-reductase was separated from endogeneous thioredoxin by hydrophobic chromatography on Phenyl Sepharose Cl 4B as described previously for the enzyme from yeast [6]. As plant extracts were heavily contaminated with phenols, the crude leaf protein collected by brief centrifugation ($18,000 \times g$, 15 min) was submitted to a second treatment with polyvinylpyrrolidone (1% g/v) in phosphate buffer as used for homogenization, but PMSF omitted and β-MSH replaced by dithiothreitol (DTT, 5 mM). The extract was filtered and solid ammonium sulphate was added to give a final concentration of 1 M. The sample was clarified by centrifugation ($24,000 \times g$, 60 min) and applied to the column equilibrated with phosphate buffered ammonium sulphate (1 M in 25 mM phosphate, pH 7.7 including EDTA 1 mM). Otherwise, the separation of enzyme and its cosubstrate was identical to methods as published before, with thioredoxin eluting at high concentrations of ammonium sulphate (≥ 0.6 M) and enzyme at low concentrations (≥ 0.1 M).

Enzyme containing samples were concentrated by precipitation with ammonium sulphate (65% saturation at 4 °C) collected by a centrifugation as before and resuspended in phosphate buffer (50 mM, pH 7.7, EDTA 1 mM, sorbitol 50 mM). Sephacryl S 300 (84×2.8 cm, equilibrated in phosphate/EDTA/sorbitol) was used for gelfiltration. The enzyme migrated with an apparent M_R of 60−70 k. Ionexchange on DEAE TSK 650 S removed phosphatase activity, the sulphate activating enzymes, APS-sulphotransferase, and residual thioredoxins. Chromatographic conditions were as outlined for the yeast PAPS-reductase. Fractions containing PAPS-reductase were made 1 M with respect to ammonium sulphate collected on a small column of Phenyl Sepharose Cl 4B and concentrated by eluting the enzyme in a step with phosphate buffered ethylenglycol (20% v/v) supplemented with EDTA 0.1 mM. Addition of ethyleneglycol was essential in order to recover the enzyme completely.

HPLC size exclusion chromatography was carried out on a Zorbax GF 450 Biosil column using phosphate buffer (50 mM, pH 7.7 supplemented with EDTA 0.1 mM, and Na_2SO_4 100 mM) at a flow rate of 0.5 ml·min^{-1}.

The enzymatic activity was assayed as thioredoxin dependent formation of [^{35}S] sulphite from [^{35}S]PAPS using dithiothreitol as auxiliary reductant. The reaction was carried out in Tris-HCl 50 mM (pH 8.0), EDTA 1 mM, NaF 25 mM, DTT 5 mM, Na_2SO_3 20 mM as carrier, [^{35}S]PAPS 3 to 18 μM (specific radioactivity 138×10^3 to 29.3×10^3 Bq·nmol^{-1}), heterologous thioredoxin 4.5 μg and enzyme in a total volume of 100 μl. Reaction conditions and determination of sulphite were as described in the assay system for the yeast PAPS-reductase.

Other methods: Preparation of substrates, determination of protein, radioactivity, and statistical methods

Recombinant thioredoxin (E. coli [14]) and spinach thioredoxins m_b, m_c, and m_d were isolated following the methods described previously [6, 15]. The thioredoxin-dependent PAPS-reductase from E. coli was employed as the indicator enzyme for thioredoxins from plants − in addition, a rapid ELISA cross-blot on nitro cellulose with antibodies raised against the recombinant thioredoxin was used for monitoring homo- or heterologous thioredoxins from the column effluents.

[^{35}S]PAPS was prepared from [^{35}S]SO_4 with the aid of a coupled ATP-sulphurylase APS-kinase enzyme system [16]. The production and purity of the nucleotides were controlled by reversed phase paired ion HPLC [17, 18].

Protein was determined by Coomassie dye binding [19]. Histograms from chromatographic curves were fitted by a cubic spline method [Interactive Microware, State Coll., P.A., U.S.A.].

Results and Discussion

PAPS-reductases from *Escherichia coli* or *Saccharomyces cerevisiae* are thioredoxin-dependent enzymes which reduce PAPS to anionic HSO_3^- and 3',5'-adenosine bisphosphate. The enzymes were found to have a high affinity (K_M in the range of 10^{-6} M) for PAPS and thioredoxin as substrates [6, 8]. Both enzymes were observed to cross-react with heterologous thioredoxins at rates between 30 to 50% of the homologous system whereas the high affinity for thioredoxin as cosubstrate was hardly affected. Moreover, the bacterial enzyme was useful as an indicator enzyme for spinach thioredoxins of the *m* type facilitating their purification to homogeneity [15]. If in deed, plants contained a similar thioredoxin-dependent PAPS-reductase it would be possible to detect the enzyme dependent sulphite formation from PAPS using heterologous thioredoxin as cosubstrate.

Gel filtration of a leaf extract from which pigments and phenols were removed as described in Materials and Methods (Fig. 1) separated the PAPS-reductase from the endogeneous thioredoxins. Its elution volume corresponded to a native molecular mass of 60 to 70 k. Omission of the DTT treatment resulted in a shift of the elution position towards a higher molecular weight (260 k). As the formation of sulphite may also have arisen from APS generated by coeluting PAPS-specific nucleotidases [20] and not from a thioredoxin dependent PAPS-reductase, the leaf protein was further purified by hydrophobic interaction chromatography, gel filtration and ion exchange chromatography recording the removal of sulphate activating enzymes, APS-sulphotransferase-, and 3'-nucleotidase activity as well as endogeneous thioredoxins.

The PAPS-reductase purified accordingly was rechromatographed on an HPLC molecular sieve and analyzed for thioredoxin dependent sulphite formation from PAPS (Fig. 2) using homologous thioredoxin (type *m* from spinach) or heterologous (recombinant protein from *E. coli*). The enzymatic activity of both assay systems coeluted suggesting that the reaction was catalyzed by the same enzyme. The molecular weight of the plant PAPS-reductase as estimated by HPLC size exclusion appeared to be 72 k confirming the value found by gel filtration (Fig. 1). PAPS-reductases from *E. coli* or *S. cerevisiae* were similar in their weight [6, 8]. The apparent rates as obtained with the heterologous thioredoxin were
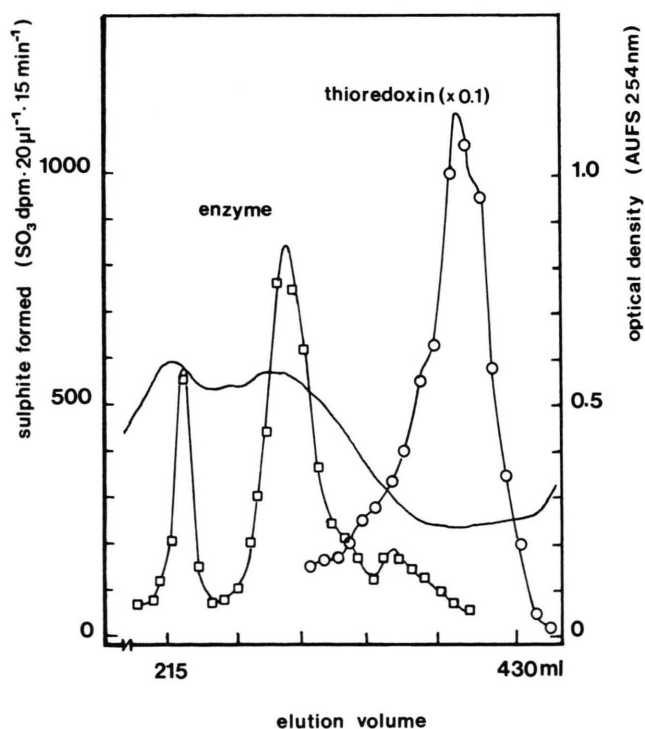

Fig. 1. PAPS-reductase and thioredoxins from spinach leaves. Chromatography on Sephacryl S 300 of a crude leaf protein extract (appr. 160 mg protein) as used for the isolation of enzyme and thioredoxins. Heterologous assays with auxiliary enzyme or cofactor from *E. coli*: □, thioredoxin and ○, PAPS-reductase added.
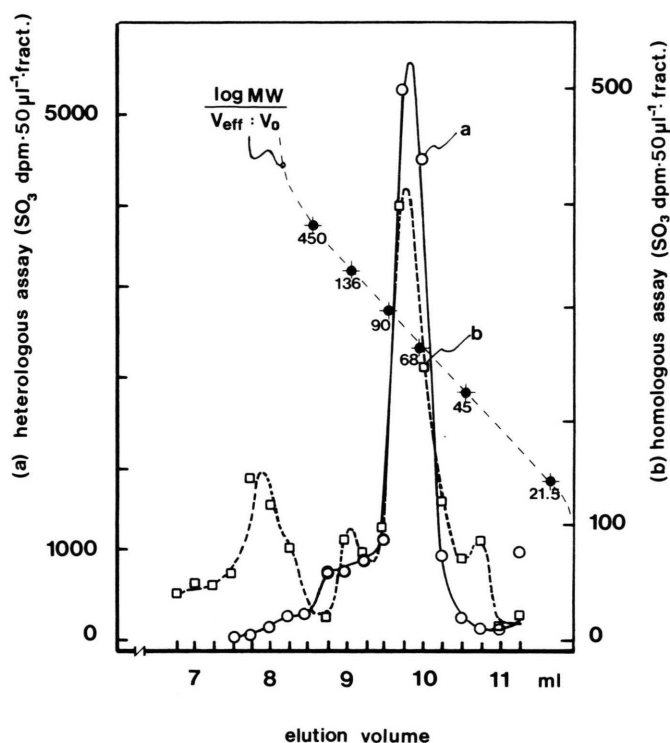
Fig. 2. Molecular weight of the PAPS-reductase determined by HPLC size exclusion. Allocation of the enzymatic activity using partially purified thioredoxin m (homologous assay: b) and homogeneous thioredoxin from *E. coli* (heterologous assay: a) as reductant. The elution volume corresponds to a molecular mass of 74–72 k as indicated by the calibration curve for the Biosil GF 450 HPLC column.

considerably higher (by a factor of 10^3). Most likely, this may have been due to a lower concentration of the plant thioredoxin m which was not homogeneous but consisted of thioredoxin m_b, m_c, and m_d. The true rate of sulphite formation will have to be established with the thioredoxin which exhibits the highest specific activity. Preliminary data suggest that the more acidic thioredoxins (the m type m_c or m_d) were preferred.

The requirement of the PAPS-reductase for thioredoxin was confirmed (Table I) using homologous thioredoxin m fraction (as above). The enzyme was inactive in the absence of its cosubstrate. The reaction had a pH optimum ranging from pH 8.0 to pH 8.4. The pH optimum when using the thioredoxin from *E. coli* (data not shown) was virtually identical. In its optimal pH the enzyme resembled the bacterial PAPS-reductase (pH 7.8 to 8.2); the enzyme from yeast differed markedly with a pH optimum of 9.2 to 9.6 [unpublished data].

The occurrence of a thioredoxin-dependent PAPS-reductase in plants like spinach, cabbage *(Brassica pekinensis)* and tobacco cell cultures (unpublished) supports the view that assimilation of inorganic sulphate in S-autotrophs follows a widely identical se-

Table I. Requirement for thioredoxin and pH optimum of the spinach PAPS-reductase. Partially purified enzyme (protein as obtained after 2. Phenyl Sepharose Cl 4B dialyzed an rechromatographed on a TSK 5PW HPLC ion exchanger) was used to reduce PAPS with enriched thioredoxin m as reductant, reaction conditions as outlined for the heterologous assay (Materials and Methods, except incubation 30 min). Identical assay for pH-dependent reactions but buffers applied at 100 mM.

	[dpm]	[nmol mg^{-1} min^{-1}]
Complete	5489	0.94
Thioredoxin omitted	271	0.046
pH 5.5 (acetate)	336	0.058
pH 6.8 (imidazol)	1145	0.196
pH 7.0 (imidazol)	3372	0.578
pH 7.4 (phosphate)	4762	0.817
pH 8.0 (tris-HCl)	5313	0.911
pH 8.4 (tris-HCl)	5299	0.909
pH 8.6 (tris-HCl)	5294	0.908
pH 9.6 (glycine-NaOH)	4804	0.824

quence of enzymatic steps. According to the data available, thioredoxin would act in two different but strictly coupled reactions: (a) by controlling the rate of PAPS-formation through modulation of the APS-kinase activity [11], and (b) by providing the electrons for the reduction of PAPS to sulphite in the

reaction of the PAPS-reductase. Moreover, the finding of this enzyme in plants questions the hypothesis that "carrier bound sulphite" is a specific intermediate formed during sulphate assimilation in plants [1, 21] because its reaction mechanism, as shown for the more thoroughly investigated enzyme from yeast, is sequential (presumably ping-pong uni-bi [6]), excluding S:sulphothioredoxin as intermediate. In the earlier literature a "bound sulphite" of the structure of a Bunte salt (RS:SO$_3$H) was described as the reaction product of a sulphotransferase [22] instead of free sulphite. As most of these previous investigations were made with partially purified enzymes or cell extracts it seems pertinent to state that the enzyme fractions may have contained disulphides which are suitable oxidants for sulphite:

$$RSSR + SO_3H \leftrightarrow RS:SO_3^- + RSH.$$
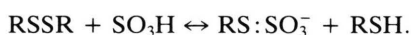
Formation of the bound sulphite RS:SO$_3$H is exergonic ($\Delta G' = 6.5$ kcal/mol) because $\Delta E^{o'}$ of the corresponding redox-reactions is $+142$ mV. Thus, bound sulphite formations from sulphite may occur spontaneously. With partially purified enzymes or intact chloroplasts, disulphides are common contaminants and their occurrence will consequently lead to the formation of bound sulphite(s). Yet, formation of S:sulphoglutathione (GS:SO$_3$H) in algae [23] could also bear some physiological significance,

though not as an intermediate, but as storage pool of sulphite because chloroplasts not only contain high concentrations of glutathione but also glutathione reductase which is reported to reduce S:Sulphoglutathione to glutathione and sulphite [24].

Sulphite as a product of the PAPS-reductase would be metabolized by the ferredoxin-dependent sulphite reductase. This enzyme has amply been demonstrated [9, 10]. It reduces free sulphite to sulphide with ferredoxin as reductant but the pool of sulphite is to be expected very small. The enzyme's low affinity constants for sulphite and ferredoxin (10^{-6} M) would scarcely allow sulphite to accumulate. Moreover, since reduced ferredoxin exerts a control over the level of reduced thioredoxin *via* the recently discovered ferredoxin:thioredoxin oxireductase [25], the APS-kinase and PAPS-reductase will only be active when sulphite can be reduced by the sulphite reductase. Thus, sulphite formation and reduction appear strictly controlled by the light reactions of photosynthesis.

[1] J. A. Schiff and H. Fankhauser, in: Biology of Inorganic Nitrogen and Sulfur (A. Trebst and H. H. Bothe, eds.), pp. 153–168, Springer Verlag, Berlin, Heidelberg, New York 1981.

[2] W. A. Abrams and J. A. Schiff, Arch. Mikrobiol. **94**, 1–10 (1983).

[3] A. Schmidt, Arch. Mikrobiol. **84**, 77–86 (1972).

[4] A. Schmidt, Planta **30**, 257–263 (1976).

[5] L. G. Wilson and D. Bierer, Biochem. J. **158**, 255–270 (1976).

[6] J. D. Schwenn, F. A. Krone, and K. Husmann, Arch. Microbiol. **150**, 313–319 (1988).

[7] M. L. S. Tsang, J. Bacteriol. **146**, 1059–1066 (1981).

[8] M. L. S. Tsang, in: Thioredoxins – Structure and Functions (P. Gadal, ed.), pp. 103–111, Editions CNRS, Paris 1983.

[9] J. Aketagawa and G. Tamura, Agric. Biol. Chem. **44**, 2371–2378 (1980).

[10] R. J. Krueger and L. M. Siegel, Biochemistry **21**, 2892–2904 (1982).

[11] J. D. Schwenn and U. Schriek, FEBS Lett. **170**, 76–80 (1984).

[12] B. B. Buchanan, Ann. Rev. Plant Physiol. **31**, 341–374 (1980).

[13] A. Holmgren, Ann. Rev. Biochem. **54**, 237–271 (1985).

[14] C. A. Lunn, S. Kathju, B. J. Wallace, S. R. Kushner, and V. Pigiet, J. Biol. Chem. **259**, 10469–10474 (1984).

[15] J. D. Schwenn and U. Schriek, Z. Naturforsch. **42c**, 93–102 (1987).

[16] U. Schriek and J. D. Schwenn, Arch. Microbiol. **145**, 32–38 (1986).

[17] H. G. Jender and J. D. Schwenn, Arch. Microbiol. **138**, 9–14 (1984).

[18] J. D. Schwenn and H. G. Jender, J. Chromatogr. **193**, 285–290 (1980).

[19] M. M. Bradford, Anal. Biochem. **72**, 248–254 (1976).

[20] M. L. S. Tsang and J. A. Schiff, Eur. J. Biochem. **65**, 113–121 (1976).

[21] J. D. Schwenn and A. Trebst, in: The Intact Chloroplast (J. Barber, ed.), pp. 315–334, Elsevier/North-Holland Biomedical Press, Amsterdam 1976.

[22] M. L. S. Tsang and J. A. Schiff, Plant Cell Physiol. **17**, 1209–1220 (1976).

[23] E. E. Goldschmidt, M. L. S. Tsang, and J. A. Schiff, Plant Sci. Lett. **4**, 293–299 (1975).

[24] B. Mannervik and K. Axelsson, in: Functions of Glutathione in Liver and Kidney (H. Sies, A. Wendel, eds.), pp. 148–163, Springer Verlag, Berlin, Heidelberg, New York 1978.

[25] B. B. Buchanan, in: Thioredoxin and Glutaredoxin Systems (A. Holmgren, C. I. Bränden, H. Jörnvall, and B. M. Sjöberg, eds.), pp. 233–242, Raven Press, New York 1986.