

A new role for thioredoxin in assimilatory sulphate reduction

Activation of the adenylylsulphate kinase from the green alga *Chlamydomonas reinhardtii* CW 15

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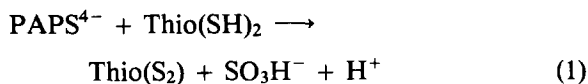
Received 6 February 1984

The activity of isolated APS-kinase (EC 2.7.1.25) from the green alga *Chlamydomonas reinhardtii* CW15 was stimulated by catalytic amounts of spinach thioredoxin f. The stimulation amounted to a factor of 3 in the presence of excess reducing thiols. A regulatory function of thioredoxin f was deduced from two observations: (a) the enzyme supported a low basal rate of PAPS formation in the absence of its effector, and (b) the saturation of the enzyme activity by thioredoxin f was sigmoidal. No evidence has been obtained of thioredoxin f serving as in the reduction of PAPS to sulphite in this alga. The slight increase in sulphite formation as observed with enriched spinach thioredoxin m is assumed to be due to a residual APS-kinase contaminating the partially purified sulphy-nucleotide unspecific sulphytransferase.

Enzyme activation *ATP:adenylylsulfate 3'-phosphotransferase* *Thioredoxin*
Assimilatory sulfate reduction *Chlamydomonas reinhardtii*

1. INTRODUCTION

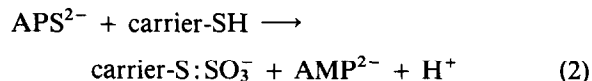
Thioredoxins are known to have multiple functions: they serve as regulatory proteins, subunits of complex enzymes, cofactors or hydrogen donors (survey [1]). In the assimilatory reduction of sulphate by the enterobacteria, thioredoxin is used as hydrogen donor in the reduction of PAPS to sulphite [2,3]:



For most of the microorganisms investigated, a regeneration of thioredoxin is assumed to involve

Abbreviations: APS-kinase, ATP:adenylylsulphate 3'-phosphotransferase (EC 2.7.1.25); APS, adenylyl 5'-phosphosulphate; DTE, dithioerythritol; DTT, dithiothreitol; GSH, reduced glutathione; PAPS, 3'-phosphoadenylyl 5'-phosphosulphate; Thio, thioredoxin

a pyridine nucleotide as reductant. Plants or green algae have been proposed to contain a different enzymatic mechanism for the reduction of sulphate employing carrier-bound intermediates (review [4]). These organisms use APS instead of PAPS as the donor of the sulphonyl group [5]:



This reaction is catalyzed by an APS-sulphytransferase activity which exhibits a high affinity for APS as substrate [6]. In analogy to the bacterial reduction mechanism, thioredoxin has been assayed as a possible sulphite carrier [7]. The increased amount of sulphite as observed in the presence of thioredoxin is difficult to assess because a homogeneous enzyme from plants or algae has not yet been obtained. In fact, crude preparations were observed to be contaminated with the enzyme APS-kinase (cf. [8]) which also

metabolizes APS:



This enzyme has been purified to homogeneity from the green alga *Chlamydomonas reinhardtii* CW15. The enzyme activity was increased in the presence of exogenous mono- and dithiols [9]. Since thioredoxin can serve as a physiological redox-active dithiol, we investigated the effect of spinach thioredoxins on the activity of the purified enzyme. The activation of APS-kinase as observed in vitro is compared with its possible role as a reductant in the sulphotransferase reaction (eq.2) with either APS or PAPS as substrate.

2. MATERIALS AND METHODS

The APS-kinase was purified from *C. reinhardtii* CW15 as in [9]. The second chromatofocussing step was omitted, using a small column of Sepharose 6B for the desalting instead. The enzyme was assayed by determination of the product PAPS formed from the substrate APS. Low rates required the use of ^{35}S -labelled APS which was prepared enzymatically as in [9]. For the detection and separation of the product HPLC was used [10] in combination with an on-line radio detector (IM 2000, Isomess, FRG) allowing the direct quantitation of the labelled nucleotides. The activities of APS- or PAPS-sulphotransferases were assayed by trapping the volatile sulphur dioxide after acidification into triethylamine as in [6]. The enzymatic activity required for the liberation of sulphite from either APS or PAPS was prepared as in [6]; the enzyme specific for PAPS, however, was purified from the same preparation including the removal of glutathione reductase as in [11] and the specific desorption of the desired protein from a Blue Sepharose CL 6B gel (unpublished).

Thioredoxins were purified from spinach leaf material as in [12] as modified in [13]. Separation of f- and m-type thioredoxin was done by chromatography on Blue Sepharose 6B which retained thioredoxin f but excluded thioredoxin m fractions. Gel filtration on Sephacryl S 200 was used to purify further the thioredoxin f while fractions of the m-type were enriched by rechromatography on hydroxyapatite (Biogel P4 HPTH, BioRad). Their functional activity was monitored by activation of fructose-1,6-bisphosphatase (EC

3.1.3.11), isolated from spinach as in [12] or, in the case of thioredoxin m, as the activation of NADP-malate dehydrogenase as in [13] from the same source. A final desalting step was included employing Sephacryl S 200. SDS-polyacrylamide gel electrophoresis as in [14] was used to record the degree of protein purification. For the determination of protein concentrations the method of [15] was used employing bovine albumin as reference.

3. RESULTS

Spinach thioredoxin f was found to activate the purified APS-kinase from *C. reinhardtii* CW15. This activation has even been obtained in the presence of saturating concentrations of thiols (DTT, DTE or GSH at 5 mM) which the enzyme was described to require for its enzymatic mechanism [9]. With either DTT or glutathione,

Table 1

Activation by thioredoxin f of the APS-kinase (EC 2.7.1.25) from *Chlamydomonas reinhardtii* CW15

	APS-kinase activity	
	PAPS (nmol/assay)	%
(I) Auxiliary reductant DTT		
Thio f, DTT, Mg^{2+} , enzyme	74	100
DTT omitted	8.1	11
Mg^{2+} omitted	11.5	14
Thio f omitted	36.4	49
Enzyme omitted	0	0
Enzyme residual activity	3.6	5
(II) Auxiliary reductant GSH		
Thio f, GSH, Mg^{2+} , enzyme	30.1	40
Thio f omitted	16.6	21

Reaction conditions: 25 mM imidazole-HCl (pH 6.8), 2 mM Mg^{2+} , 5 mM dithiothreitol, or reduced glutathione, 0.03 mM ATP, 4.4 nM APS (spec. act. 677 Bq/nmol), 1.5 μg purified APS-kinase and thioredoxin f (spinach) 3.0 μg protein in a volume of 0.250 ml. The enzyme was activated for 30 min in the presence of thioredoxin and DTT (or GSH) as the reductant at 30°C before the reaction was started by the introduction of the substrate APS and assayed for another period of 7 min

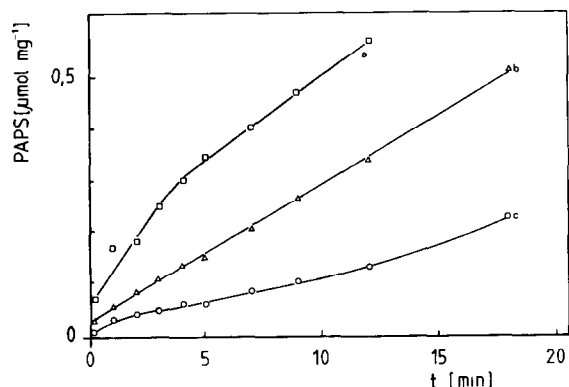


Fig.1. Reaction kinetics of *C. reinhardtii* CW15 APS-kinase: increase of initial rate of enzyme due to the presence of thioredoxin f. (a) Complete assay including thioredoxin f (from spinach) and DTT, (b) thioredoxin f omitted, and (c) basal rate of enzyme in the absence of thiols. Concentration of reactant as in legend to table 1. The reaction was started after temperature equilibration (5 min at 30°C) by the introduction of [35 S]APS. Samples were withdrawn at intervals as indicated and assayed for [35 S]PAPS by reversed-phase ion-pair HPLC [9].

the amount of the reaction product PAPS was increased when thioredoxin f was included in the assay (table 1). The kinetics of PAPS formation showed that thioredoxin f had increased the velocity of the enzyme in the initial phase of the reaction

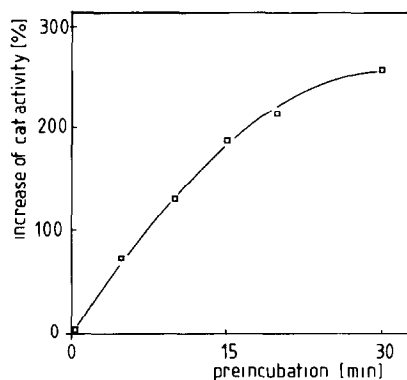


Fig.2. Time course of APS-kinase activation by thioredoxin f: the enzyme (1.5 μ g protein) was preincubated with thioredoxin f (3 μ g) and DTT (5 mM) for periods as indicated. After preincubation the individual samples were complemented with APS and assayed for 5 min under conditions as specified in the legend to table 1.

by a factor of 2.6 (fig.1). This increased rate can be maintained for periods exceeding 4–5 min when the enzyme is preincubated with DTT and thioredoxin f together before APS is introduced to start the reaction. A period of 30 min was sufficient to saturate the activating effect by thioredoxin f (fig.2), increasing the rate by a factor of 3. By maintaining these experimental conditions, the amount of thioredoxin f was varied for two fixed concentrations of substrates (trace a and b in fig.3). In both cases approx. 2 μ g thioredoxin f were required to activate the enzyme fully. The stimulation by thioredoxin f of PAPS formation at high concentrations of APS (trace b) is noteworthy because this range of substrate concentration has been found to be inhibitory when the enzyme is assayed in the absence of thioredoxin [9].

The APS-kinase appeared to present the only enzyme of the plant sulphate activating mechanism which is activated by thioredoxin f because the ATP-sulphurylase and inorganic pyrophosphatase showed no change in activity when assayed in the presence of the thioredoxin system (not shown).

In view of the hydrogen donor or sulphite carrier molecule, participation of thioredoxin f or m in the formation of sulphite (according to eq.1,2) from either PAPS or APS has been investigated with partially enriched sulphotransferase fractions from *C. reinhardtii* CW15. The enzyme fraction which used both substrates (e.g., APS and PAPS) was

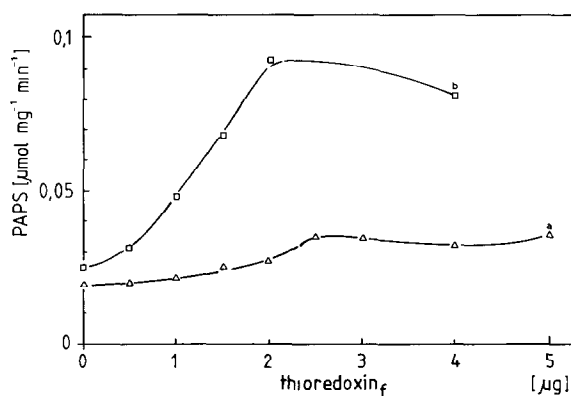


Fig.3. Saturation, by thioredoxin f, of APS-kinase at different concentrations of the substrates APS and ATP: activation period 30 min, reaction initiated by the introduction of the substrate APS. (a) 4.4 μ M APS (30 μ M ATP), and (b) 8.8 μ M APS (300 μ M ATP).

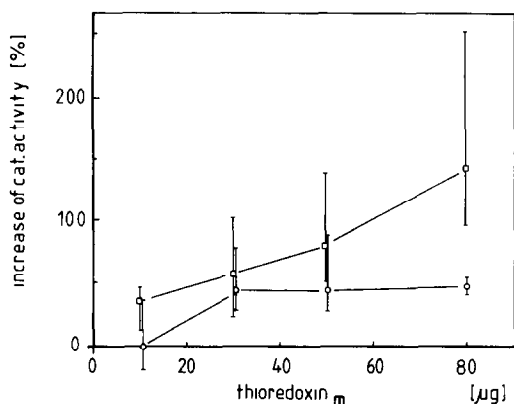


Fig.4. Effect by thioredoxin m (spinach) upon enriched sulphotransferases from *C. reinhardtii* CW15 (assayed as formation of sulphite from APS (□) or PAPS (○)). The transferase activity was measured as in [6] in the presence of DTE (2 mM) as reducing thiol; other conditions were: 25 mM Tris-HCl (pH 7.9), 2.5 mM MgCl₂, 14.6 μM [³⁵S]APS (spec. act. 1601 Bq/nmol), 9.5 μg enriched nucleotide unspecific sulphotransferase in a total volume of 250 μl. The increase is given as the mean value of triplicates; rate of control, 18.8 nkat/mg protein (SE 31.8%). The PAPS-specific sulphotransferase activity was measured under identical conditions, except for the use of [³⁵S]PAPS as substrate (29 μM, same specific activity as [³⁵S]APS) and purified PAPS-sulphotransferase (19 μg protein) (rate of control: 3.95 nkat/mg, SE 16.1%). Concentrations of thioredoxin m were varied as indicated.

prepared and assayed as in [6] by the liberation of sulphite employing DTT as the reductant and acceptor thiol at saturating concentrations. Supplementation of this reaction with thioredoxin f at concentrations sufficient to activate APS-kinase (see above) did not increase the amount of sulphite from APS or PAPS. When thioredoxin m was used instead of the f-type, the amount of sulphite formed from APS was increased by 150% (mean value of 3 experiments, fig.4). With PAPS as substrate in combination with a PAPS-specific sulphotransferase (separated from the sulphonucleotide unspecific enzyme by rechromatography on 2',5'ADP Sepharose and Blue Sepharose CL 6B) the amount of sulphite was increased marginally (46%) in the presence of thioredoxin m. Thioredoxin f did not affect the formation of sulphite under these conditions.

4. DISCUSSION

Thioredoxins from plants have attracted much attention because of their subtle contribution to the regulation of enzymes involved in photosynthetic carbon dioxide fixation [16]. As shown in this investigation of the assimilatory sulphate reduction by the green alga *C. reinhardtii* CW15, the function of thioredoxin f can be described as that of an activator of the APS-kinase. In comparison to the residual enzyme activity observed in the absence of thiols, the thioredoxin/DTT system increased the rate by a factor of 20. The specificity of the activation by thioredoxin is illustrated by the finding that, even in the presence of saturating amounts of DTT, the addition of thioredoxin f led to an additional increase of the rate. The physiological relevance of the activation of APS-kinase by thioredoxin can be seen in a regulation between sulphate assimilation and light-driven ATP formation and provision of reductants such as ferredoxin or NADPH. Since in chloroplasts the reduction of thioredoxin is linked to the reduction of ferredoxin, increased APS-kinase activity as caused by thioredoxin enables sulphate assimilation to benefit directly from photosynthesis.

Considering the function of thioredoxins as a hydrogen donor or sulphite carrier (eq.1,2) only thioredoxin m gave rise to increased sulphite formation. Since this stimulation was virtually restricted to the partially enriched sulphotransferase when APS was used as substrate and thioredoxin supplied in excess amounts, the significance of this finding remains doubtful. Moreover, due to contamination of this enzyme fraction with APS-kinase (unpublished) the stimulation of sulphite formation could also have been due to increased kinase activity. Yet, the finding that the amount of sulphite formed from PAPS did not change considerably in the presence of either thioredoxin could have been deduced from the inadequacy of the sulphotransferase assay. In fact, this assay includes excessive amounts of unphysiological thiols (DTT or DTE, which are used for the reduction of thioredoxin) that may compete with thioredoxin as an inhibitory substrate analogue. The data as presented do not exclude a participation of thioredoxin in the reduction of PAPS to sulphite sulphur similar to the mechanism of reduction in microorganisms [2,3]. They show, however, that

results obtained with only partially purified sulphotransferases are insufficient to prove participation of thioredoxin in assimilatory sulphate reduction by algae. Future work, therefore, will have to employ not only a homogeneous transferase, but also the enzymatic thioredoxin reducing system. Yet, from the present results it appears safe to state that the APS-kinase from *C. reinhardtii* CW15 is activated by thioredoxin f.

ACKNOWLEDGEMENT

The financial support of this work by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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