Regulation of 2-carboxy-D-arabinitol 1-phosphate phosphatase: activation by glutathione and interaction with thiol reagents

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2-Carboxy-D-arabinitol 1-phosphate (CA1P) phosphatase degrades CA1P, an inhibitor associated with the regulation of ribulose bisphosphate carboxylase/oxygenase in numerous plant species. CA1P phosphatase purified from *Phaseolus vulgaris* was partially inactivated by oxidizing conditions during dialysis in air-equilibrated buffer. Phosphatase activity could then be stimulated 1.3-fold by dithiothreitol and also by addition of reduced thioredoxin from *Escherichia coli*. These effects were enhanced synergistically by the positive effector, fructose 1,6-bisphosphate (FBP). Most notably, CA1P phosphatase activity was stimulated up to 35-fold by glutathione, and was sensitive to the ratio of reduced (GSH) to oxidized (GSSG) forms. At concentrations of glutathione approximating measured levels in chloroplasts of *P. vulgaris* (5 mM total S), CA1P phosphatase exhibited > 20-fold stimulation by a change in the redox status of glutathione from 60 to 100 % GSH. This stimulation was augmented further by reduced *E. coli* thioredoxin. In contrast, FBP, which activates CA1P phosphatase under reducing conditions, was strongly inhibitory in the presence of GSSG. We propose that glutathione may have an appreciable role in the light/dark regulation of CA1P phosphatase *in vivo*. A model for the reversible activation of CA1P phosphatase by GSH was derived based upon the various responses of the enzyme's activity to a range of thiol reagents including *N*-ethylmaleimide, 5,5′-dithiobis-(2-nitrobenzoic acid) and arsenite. These data indicate that the bean enzyme contains two physically distinct sets of thiol groups that are critical to its redox regulation.

Key words: carbon metabolism, *Phaseolus*, photosynthesis, redox regulation, Rubisco.

INTRODUCTION

2-Carboxy-D-arabinitol 1-phosphate (CA1P) is a naturally occurring, tight-binding inhibitor of ribulose bisphosphate carboxylase/oxygenase (Rubisco) that accumulates in the chloroplasts of many plant species during darkness or at low irradiance [1,2]. CA1P participates as an integral component of the multiple mechanisms regulating catalysis by Rubisco [3,4]. In the light, Rubisco activase mediates activation of Rubisco at physiological levels of CO_2 and facilitates dissociation of CA1P from Rubisco [5]. Unbound CA1P is subsequently hydrolysed to carboxy-D-arabinitol and inorganic phosphate (P_i) by CA1P phosphatase [6,7]. The accumulation of CA1P to levels that exceed the active site concentration of Rubisco in the chloroplast compartment of *Phaseolus vulgaris* leaves [8,9] indicates that CA1P phosphatase is inactivated in the dark. However, the mechanism responsible for light/dark regulation of CA1P phosphatase has not been fully elucidated. It is known that this chloroplast enzyme is activated by certain Calvin-cycle intermediates, including D-fructose 1,6-bisphosphate (FBP), D-ribulose 1,5-bisphosphate and 3-phosphoglyceraldehyde, and inhibited by free phosphate [10]. Sensitivity to prevailing levels of these effectors could certainly contribute to the regulation of CA1P phosphatase *in vivo* [10,11]. It is well established that other light-activated chloroplast phosphatases are subject to redox regulation mediated by thioredoxin [12]. For example, the activities of fructose and sedoheptulose bisphosphatases are linked to chloroplast electron transport via the ferredoxin–thioredoxin system [13,14]. The photosynthetic generation of high levels of oxygen in the chloroplast provides conditions leading to the inhibitory re-oxidization of susceptible enzymes if they are not continuously kept active by the opposing electron-transport-mediated reduction [15].

There have been two observations indicating that CA1P phosphatase activity may also be modulated by this type of redox mechanism. First, degradation of CA1P during illumination is dependent upon a functional photosynthetic electron transport system: 3-(3,4-dichlorophenyl)-1,1-dimethylurea treatment of darkened *P. vulgaris* leaf discs [16], and Methyl Viologen-treatment of tobacco protoplasts [17], are both capable of preventing the light-dependent recovery of Rubisco activity inhibited by CA1P. These results indicate that CA1P degradation occurs only when there is an uninterrupted passage of electrons to the reducing side of photosystem I. Second, CA1P phosphatase from tobacco or bean leaves has shown a requirement for dithiothreitol (DTT) to maintain the enzyme in an active state. Dialysis in the absence of this reducing agent caused a reversible loss of enzyme activity [10,18]. Both observations are consistent with a potential redox regulation of CA1P phosphatase either by the thioredoxin system or by a related mechanism.

The presence of relatively large pools ($\leqslant 20$ mM) of the monothiol glutathione in chloroplasts of many plants has been linked to its function as a redox buffer with a role in detoxifying activated oxygen species [19]. However, the activity of certain thioredoxin-regulated chloroplast enzymes can be modulated by the ratio of reduced versus oxidized glutathione ([GSH]/[GSSG]). This has been observed *in vitro* with NADP-dependent malate dehydrogenase from pea, either in native [20] or truncated form [21]. In addition, a direct activation of malate dehydrogenase by GSH was previously reported using partially purified enzyme from several other species of C_3 plants, including barley, spinach and wheat [22].

Abbreviations used: CA1P, 2-carboxy-D-arabinitol 1-phosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; FBP, D-fructose 1,6-bisphosphate; hGSH, reduced homoglutathione; hGSSG, homoglutathione disulphide; NEM, *N*-ethylmaleimide; *p*CMB, *p*-chloromercuric benzoic acid; Rubisco, D-ribulose bisphosphate carboxylase/oxygenase.

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In this study using purified CA1P phosphatase, we have investigated the regulatory effects of the naturally occurring thiol reducing agents, thioredoxin and glutathione, as well as the interaction of the enzyme with DTT and the known effectors FBP and P_i. We also examined the activity of CA1P phosphatase in the presence of a variety of thiol-group modifying agents, and developed a model for the regulation of the enzyme based on the kinetic data obtained.

EXPERIMENTAL

Plant materials

Beans (*P. vulgaris* L. cv. Linden) were grown in commercial compost in a greenhouse at 20–25 °C under natural light supplemented with mercury halide lamps to give a 16 h photoperiod. For purification of CA1P phosphatase, fully expanded leaflets were excised from 4-week-old plants after 12 h in the light, and pulverized in liquid nitrogen prior to storage at −80 °C.

Synthesis of [2-^{14}C]CA1P

D-Ribulose 1,5-bisphosphate was synthesized from ribose 5-phosphate, and [2-^{14}C]CABP was synthesized from the reaction of D-ribulose 1,5-bisphosphate and [^{14}C]KCN [23,24]. [2-^{14}C]CA1P was prepared by limited hydrolysis of [2-^{14}C]carboxy-D-arabinitol bisphosphate and purified as described by Moore et al. [8].

Purification of CA1P phosphatase

CA1P phosphatase was purified from bean plants essentially as described by Kingston-Smith et al. [11]. Several modifications to this method were made in the later stages of the purification after sucrose density-gradient centrifugation and subsequent gel filtration of the enzyme preparation using Sephadex G-25. A concentrated protein sample (180 mg of protein in 20 ml) was loaded on to a Dyematrix Green A column (2.5 × 13.5 cm; Amicon, Danvers, MA, U.S.A.) at 0.33 ml/min with 50 mM Tris/HCl (pH 7.5)/1 mM EDTA/10 mM $MgCl_2$/1 mM $CaCl_2$/10 mM $NaHCO_3$/2 mM DTT (buffer A) and allowed to stand for 1 h. To maximize interaction of extracted proteins with the column dye, the pumping direction was alternated six times for 5 min periods before eluting the protein from the column with a linear 0–1.5 M KCl gradient in the same buffer at 0.33 ml/min. Fractions (2 ml) containing CA1P phosphatase activity eluted at between 0.07 and 0.1 M KCl and were pooled prior to bringing the solution to 80 % saturation with $(NH_4)_2SO_4$. After centrifugation at 12000 ***g*** for 20 min, the pellet was dissolved in 3 ml of buffer A and passed through a Sephacryl S-300 column (1.3 × 61 cm) with a flow rate of 0.08 ml/min. Peak fractions with CA1P phosphatase activity were pooled, concentrated by precipitation with 80 % $(NH_4)_2SO_4$ and centrifuged at 20000 ***g*** for 20 min. The pellet was dissolved in 2.6 ml of buffer A, and 0.75 ml of the sample was dialysed overnight at 4 °C against four changes of air-equilibrated buffer containing 50 mM Tris/HCl (pH 7.5)/1 mM EDTA/4 mM DTT (buffer B). The remaining volume of protein was dialysed in the same way against the same buffer, but excluding DTT (buffer C). Each of the dialysed enzyme solutions (designated 'Green-column enzyme') were kept frozen in liquid nitrogen until further use. In a final purification step, 0.9 mg of CA1P phosphatase dialysed with buffer C was loaded on to an FPLC Mono Q column and eluted with a linear 0–0.6 M KCl gradient in buffer C. Fractions containing CA1P phosphatase activity were pooled, immediately dialysed for 12 h with four changes of buffer C to remove KCl, and stored in liquid nitrogen until further use.

Assay of CA1P phosphatase activity

Unless stated otherwise, the CA1P phosphatase used in this study was purified through to the Mono Q column step. The enzyme activity was measured radiometrically as described by Holbrook et al. [7] except that the standard reaction buffer contained 50 mM Tris/HCl (pH 7.5)/1 mM EDTA. Assays (25 μl) were run with 0.5 mM [^{14}C]CA1P at 25 °C for 3 min using Mono Q-purified CA1P phosphatase, or for 10 min with the Green-column enzyme. Variations in assay procedure are indicated in the Figure legends. Thioredoxin f was a generous gift from Dr. B. B. Buchanan. Protein content was determined colorimetrically using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) and a BSA standard.

Determination of reduced homoglutathione (hGSH) and homoglutathione disulphide (hGSSG)

The *P. vulgaris* plants were grown as described above. Leaf samples comprising four fully-expanded single trifoliolate leaflets were taken after 12 h of illumination by natural and supplemental light. The corresponding leaflets from the same leaves were sampled following a subsequent 12 h period after plants were transferred to a darkened growth chamber at 23 °C. After excision, the leaflets were immediately frozen in liquid nitrogen, combined and pulverized as a batch. The levels of hGSH and hGSSG in bean leaf extracts were estimated using GSH and GSSG (Sigma, St. Louis, MO, U.S.A.) as standards [25], by correcting measured values as described by Klapheck [26].

RESULTS

Properties of CA1P phosphatase from *P. vulgaris*

CA1P phosphatase was purified to homogeneity as judged by SDS/PAGE where enzyme from the Mono Q column formed a single band at 55 kDa [11,18,27]. Modification of the purification protocol [11] by including protein-concentrating steps, and also by ensuring extra interaction of CA1P phosphatase with the dye matrix, resulted in an enhancement of protein purification from 75-fold to 174-fold with the Green-column step.

The response of CA1P phosphatase to known effectors and KCl is presented in Table 1, which shows the properties of moderately oxidized enzyme dialysed in the absence of DTT. Typically, purified enzyme exhibited an approximately two-fold decrease in activity after this treatment, but failed to show complete inactivation by removal of DTT. Addition of the positive effector, FBP, previously reported to stimulate CA1P phosphatase activity by at least 10-fold in the presence of DTT [10,11], increased activity by only 60 % with the DTT-depleted enzyme (Table 1). In contrast, 5 mM P_i, a negative effector [10], caused almost complete inhibition of Mono Q-purified CA1P phosphatase in the absence of DTT (Table 1). These results indicate that exposure of CA1P phosphatase to oxidizing conditions during dialysis with air-equilibrated buffer may modify its response to physiologically relevant effectors. Such an outcome is consistent with the general behaviour of other redox-regulated chloroplast enzymes susceptible to re-oxidation by atmospheric levels of O_2 in the absence of reducing agents [28].

The inclusion of KCl in assays had an appreciable inhibitory effect on CA1P phosphatase activity, pretreated in the absence of DTT (Table 1). This observation differs from an earlier study [11], which reported that increased ionic strength due to the addition of KCl significantly enhanced the activity of CA1P phosphatase, and obviated the need for DTT to maintain enzyme activity. In contrast, Charlet et al. [18], while not reporting an inhibitory effect of KCl on the bean enzyme, saw little or no

Table 1 Response of CA1P phosphatase activity to effectors and KCl, in the presence and absence of DTT and *E. coli* thioredoxin

Results shown are for Mono Q-purified enzyme dialysed with or without 4 mM DTT in air-equilibrated buffer, and represent means of triplicate assays. The enzyme was preincubated for 1 h with effectors before assay for 10 min with [^{14}C]CA1P (see the Experimental section). Where indicated as 'Reductant added', 20 mM DTT or 2.5 μM reduced *E. coli* thioredoxin were included in this preincubation. v_e/v_0 represents the ratio of activities in the presence (v_e) and absence (v_0) of effector. The control (v_0) value was 1.4 μmol of carboxy-D-arabinitol formed/min per mg of protein. Data in parentheses are from [10].

		CA1P phosphatase (v_e/v_0)			
	Dialysis ...	+ DTT	− DTT		
Effector or solute (mM)	Reductant added ...	None	None	+ DTT	+ Thioredoxin
Control		1.00	1.00	1.30	1.60
FBP (5.0)		(7.00)	1.61	3.10	3.80
P_i (0.5)		(0.58)			
P_i (5.0)		(0.37)	0.01	0.05	0.06
KCl (25.0)		–	0.92	–	–
KCl (200.0)		–	0.21	–	–

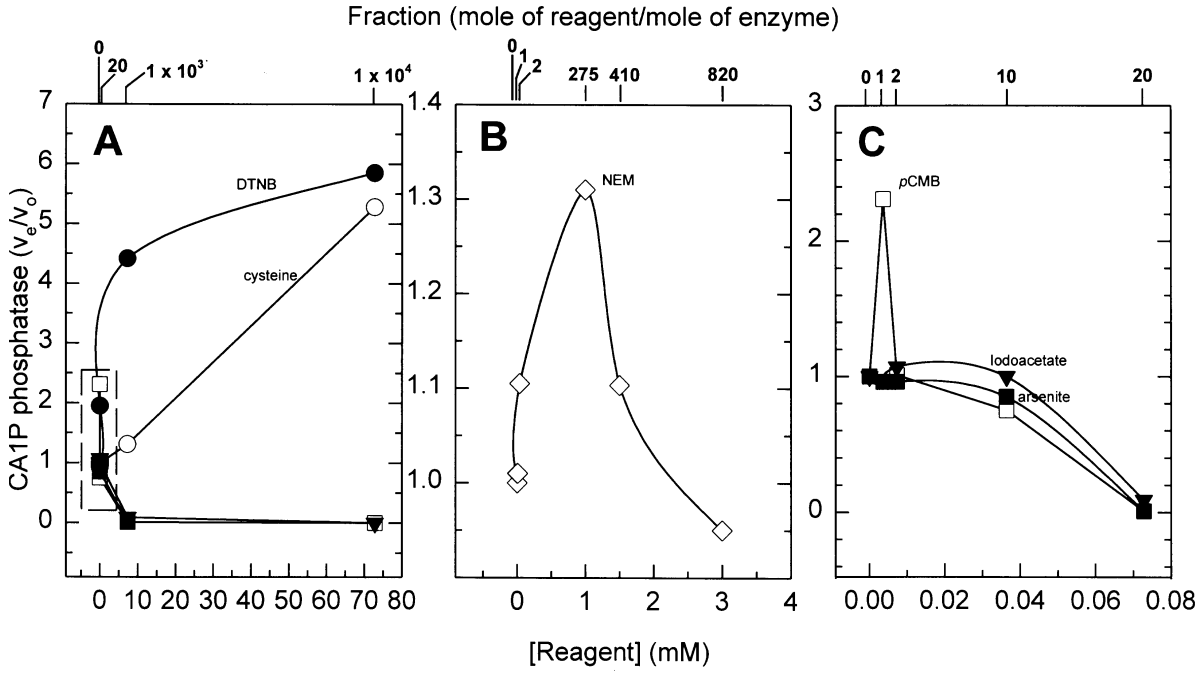


Figure 1 Response of CA1P phosphatase activity to treatment with diverse thiol reagents

(**A**, **B**) Mono Q-purified enzyme (0.05 μg), dialysed for 12 h without DTT, was incubated with the reagents indicated for 30 min prior to assay for 3 min with 0.5 mM [^{14}C]CA1P. Control activity (v_0) was 1.62 μmol of carboxy-D-arabinitol formed/min per mg of protein. (**C**) Expanded scale showing the effects of *p*CMB, iodoacetate and arsenite at the low concentrations presented within the dashed lines in (**A**). Symbols in (**A**) and (**C**) are the same.

stimulation of CA1P phosphatase by this salt. The basis for these disparate results is presently under investigation.

Interaction of CA1P phosphatase with DTT and thioredoxin

To address the possibility that CA1P phosphatase is regulated by reductive activation, and specifically by the thioredoxin system, we examined the effects of DTT and reduced thioredoxin on enzyme activity. Incubation with 20 mM DTT re-activated CA1P phosphatase partially inhibited by exposure to air oxidation during dialysis for 12 h without DTT (Table 1). However, the greatest stimulation observed was minor at 1.3-fold after 30 min. More notably, reduced *E. coli* thioredoxin activated CA1P phosphatase by more than 1.5-fold during incubations between 3 and 30 min (Table 1). This source of thioredoxin has been utilized as a substitute for native plant thioredoxins *in vitro* [29] because it is unspecific in heterologous assays [30]. The thioredoxin-mediated stimulation of CA1P phosphatase was also noted using enzyme maintained in the presence of DTT (results not shown), but was most pronounced with enzyme previously dialysed in the absence of DTT (Table 1). Equivalent concentrations of thioredoxin f from spinach had no effect on the activity of the enzyme (results not shown), despite its more negative redox potential than thioredoxin from *E. coli* [31]. In

addition, the observed stimulation of CA1P phosphatase by *E. coli* thioredoxin was appreciably less than that typically recorded in comparable experiments with FBPase and sedoheptulose bisphosphatase in the presence of thioredoxin f [32]. However, both reduced *E. coli* thioredoxin and DTT stimulated CA1P phosphatase synergistically with the positive effector FBP (Table 1). In comparison, these reductants had little effect on enzyme activity inhibited by 5 mM P_i (Table 1).

Previous work with CA1P phosphatase from tobacco showed that the positive effector FBP increased V_{max} but not the K_m (for CA1P) of the enzyme [10]. Similarly, neither DTT nor thioredoxin had a significant influence on the affinity of the enzyme for substrate: the apparent K_m for CA1P was measured as 0.237 ± 0.074 mM ($\pm$S.D.) in the presence of 0.5 μM *E. coli* thioredoxin reduced with 20 mM DTT, as compared with 0.209 ± 0.039 mM ($\pm$S.D.) in assay medium lacking either reductant.

In accord with the stimulatory effects of DTT (Table 1), other reducing agents such as cysteine increased CA1P phosphatase activity five-fold, but required high concentrations (70 mM) to produce this enhancement (Figure 1). Taken together, the data support the proposal that the observed re-activation of CA1P phosphatase is associated with reduction of thiol groups [10], as exemplified by several other light-regulated phosphatases present in the chloroplast [33,34].

Effect of thiol-modifying reagents

The effects of a diverse range of thiol-modifying reagents on CA1P phosphatase activity are shown in Figure 1. Titration of the enzyme with increasing concentrations of the thiol-blocking reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), caused a marked stimulation of activity, which did not saturate even at reagent levels representing a > 1000-fold molar excess over the CA1P phosphatase present. This unexpected result indicates that mixed disulphide formation with accessible thiol groups on the enzyme causes a strong enhancement of catalysis.

At pH 7.5 the alkylating agent, *N*-ethylmaleimide (NEM), was slightly stimulatory at low concentrations up to 1 mM, but became inhibitory at concentrations higher than a 275-fold molar excess over CA1P phosphatase (Figure 1B). A similar concentration-dependent biphasic response was observed with the metal-binding reagent *p*-chloromercuric benzoic acid (*p*CMB), which was stimulatory only at low concentrations close to molar equivalency with the protein, and resulted in complete inhibition at higher levels (Figure 1C). In contrast, arsenite, which interacts specifically with vicinal dithiols [35], was inhibitory at all levels tested.

In the lower concentration range where inhibition of CA1P phosphatase was observed (Figures 1B and 1C), the relative effectiveness of the reagents tested was as follows: *p*CMB > arsenite > iodoacetate > NEM. This ranking indicates that the more negatively charged reagents exhibit a greater reactivity with the enzyme. The region of CA1P phosphatase containing reactive thiol or disulphide groups is therefore likely to be hydrophobic and positively charged.

The kinetics of the interaction of NEM with CA1P phosphatase were analysed in more detail (Figure 2). A time course of enzyme activity during incubation with low concentrations of this reagent showed an initial activation of catalysis followed by a progressive inhibition of phosphatase activity. At higher concentrations of NEM, the onset of inhibition was more rapid. The activation constant, $^aK_{obs}$, calculated according to Blanke and Hager [36], was proportional to the concentration of NEM. The subsequent inactivation of CA1P phosphatase followed first-order kinetics with respect to the concentration of NEM if the partial activation

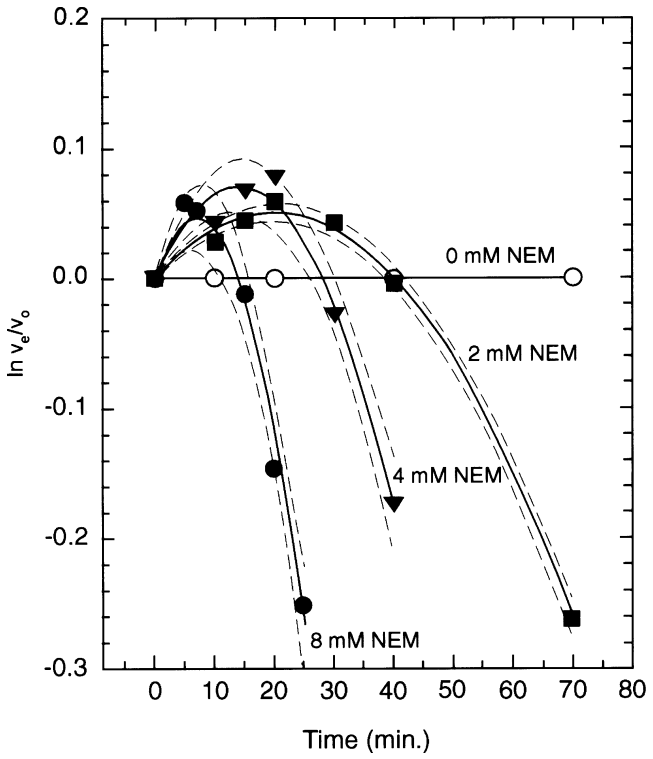


Figure 2 Kinetics of the interaction of CA1P phosphatase with NEM

CA1P phosphatase, pretreated as in Figure 1, was incubated for the times shown with a range of NEM concentrations prior to assay of CA1P phosphatase activity. A plot of $-\ln(v_e/v_o)$ against time was used to estimate the rates of initial activation and inactivation according to the integrated rate equation [36]: $-\ln(v_e/v_o) = K_{obs}\chi t$ where χ represents concentration of the reagent, t represents incubation time and K_{obs} represents the bimolecular constant. Curves were fitted using non-linear regression analysis. Broken lines represent the 95% confidence limits of the data.

at low concentrations and shorter incubation times were disregarded The inactivation constant, $^iK_{obs}$, was also found to be proportional to the concentration of NEM (results not shown), indicating a bimolecular reaction between the enzyme and NEM. The second-order activation rate constant, ^aK, and the second-order inactivation constant, ^iK, were calculated to be 0.086 $min^{-1} \cdot M^{-1}$ and 2.434 $min^{-1} \cdot M^{-1}$ respectively [36], showing a 28-fold difference in the rate of activation versus inactivation of CA1P phosphatase by NEM. The data are consistent with partial alkylation of thiol groups causing an initial activation of CA1P phosphatase, followed by inhibition of catalysis upon full alkylation of reactive thiols. Arsenite, which can bridge vicinal thiols, did not show any partial activation of CA1P phosphatase (Figure 1C). Therefore, partial activation by NEM is probably due to modification of half the susceptible thiol groups because arsenite is incapable of a two-stage modification of vicinal thiols.

Effect of GSH and GSSG

Because of the occurrence of relatively high levels of glutathione as a natural reductant in chloroplasts [37], it was of interest to examine possible effects of this tripeptide monothiol on CA1P phosphatase activity. An appreciable response would be predicted because of the strong positive influence of DTNB on the reaction rate (Figure 1A), and the similarity between DTNB and GSSG in terms of the reaction mechanisms involving mixed disulphide formation with susceptible proteins [31].

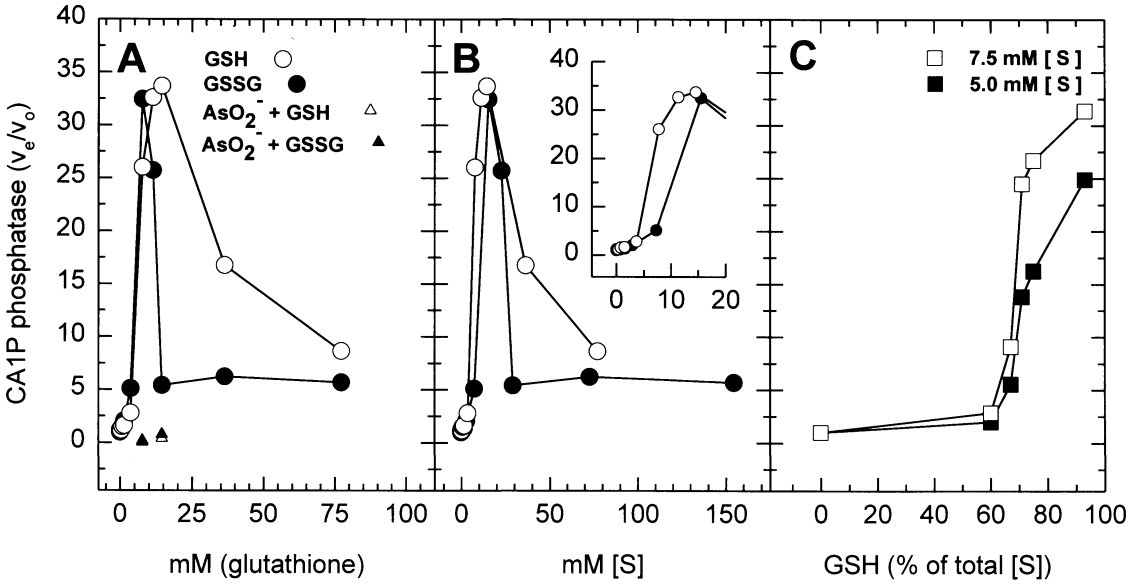


Figure 3 Effect of glutathione on CA1P phosphatase activity

(**A**) Mono Q-purified enzyme, pretreated as in Figure 1, was incubated for 10 min with or without 5 mM arsenite (AsO_2^-) prior to addition of glutathione in GSH or GSSG forms, giving the range of final concentrations shown. (**B**) Replot of data in (**A**) on the basis of the total sulphur content of GSH or GSSG. Inset: expanded scale of (v_e/v_0) at 0–20 mM S. (**C**) Activation of CA1P phosphatase by increasing the percentage of reduced glutathione: {[GSH]/([GSH] + [GSSG])} × 100 at fixed concentrations of total glutathione.

In *P. vulgaris*, homoglutathione (γ-L-glutamyl-L-cysteinyl-B-alanine) replaces glutathione (γ-L-glutamyl-L-cysteinylglycine) as the predominant (> 99 %) and analogous free thiol in leaves [26,38]. While recognizing that this difference exists with bean, the experiments reported here utilized GSH and GSSG as commercially available analogues. Figure 3(A) shows that 16 mM GSH or 8 mM GSSG caused a 33–35-fold activation of the air-oxidized bean CA1P phosphatase pretreated by extensive dialysis in the absence of DTT. This observation is important because the magnitude of stimulation far exceeded that observed with either thioredoxin or positive effectors such as FBP. Similar results with glutathione were also obtained using CA1P phosphatase isolated from tobacco leaves (results not shown). In addition, arsenite, a thiol-modifying reagent specific for vicinal dithiols [35], prevented the activation of the enzyme by GSH or GSSG (Figure 3A), as well as by DTNB (results not shown).

Maximal activation of CA1P phosphatase by GSSG required stoichiometrically one half the amount of reagent compared with GSH (Figure 3A). The mechanism of activation may therefore be dependent upon the respective sulphur content of the GSH or GSSG added, and so the same data were re-plotted taking this into account (Figure 3B). Both forms show maximal activity of CA1P phosphatase at 16 mM total sulphur. Based on this observation, we suggest that the activation of CA1P phosphatase by GSH requires 2 mol of GSH per mol of protein disulphide to give two thiols. Hence, the equivalent interaction of GSSG with the enzyme requires 1 mol of GSSG per mol of protein disulphide, giving a mixed disulphide. In this case, GSH appears to be more stimulatory than oxidized glutathione ([GSSG]/2) at concentrations between 4 and 10 mM (inset of Figure 3B). Most notably, Figure 3(C) shows that CA1P phosphatase activity is sensitive to the relative proportions of GSH and GSSG in the assay medium. At fixed total sulphur contents of 5 or 7.5 mM, the enzyme showed a striking increase in activity as the glutathione form supplied was changed from oxidized to reduced.

We also investigated the interaction of GSH and GSSG with the positive effector FBP (Figure 4A). There was an additive stimulatory effect of FBP on CA1P phosphatase with a low concentration of GSH (0.1 mM). However, at GSH concentrations over 4 mM, the addition of FBP did not enhance catalytic rates further (Figure 4A). In contrast, FBP together with either GSSG or DTNB strongly inhibited CA1P phosphatase activity (Figure 4A). Formation of a mixed disulphide with GSSG may therefore expose the active site in such a way that FBP acts as an inhibitor.

Reduced *E. coli* thioredoxin enhanced the stimulatory effect of both GSH and GSSG on activity of CA1P phosphatase previously maintained in the presence of 4 mM DTT (Figure 4B). Hence, these naturally occurring thiol reagents are also capable of stimulating activity of an enzyme preparation that had not been purposely inactivated by exposure to oxidizing conditions.

Concentrations of hGSH and hGSSG in bean leaves

As noted by Klapheck [26], leaves of *P. vulgaris* and other legumes contain a high proportion of the tripeptide homoglutathione (hGSH and hGSSG), as opposed to GSH and GSSG, which are present as a natural redox buffer in cells of most other plant species. Both [hGSH]/[hGSSG] and [GSH]/[GSSG] ratios can be measured using the same assay [25], if the appropriate standard is used, or by applying a correction factor to account for the higher sensitivity of the assay towards hGSH and hGSSG [26]. Table 2 shows that the concentration of total homoglutathione ([hGSH] + [hGSSG]) in *P. vulgaris* leaves decreases slightly during a 12 h dark period. However, if this result is analysed more appropriately with respect to the total sulphur content of hGSH plus hGSSG in the chloroplast, there is little net change as a result of illumination (Table 2). Thus, in the light, approximately 25 % more hGSH is formed from hGSSG, without a net increase in the estimated level of this tripeptide in the

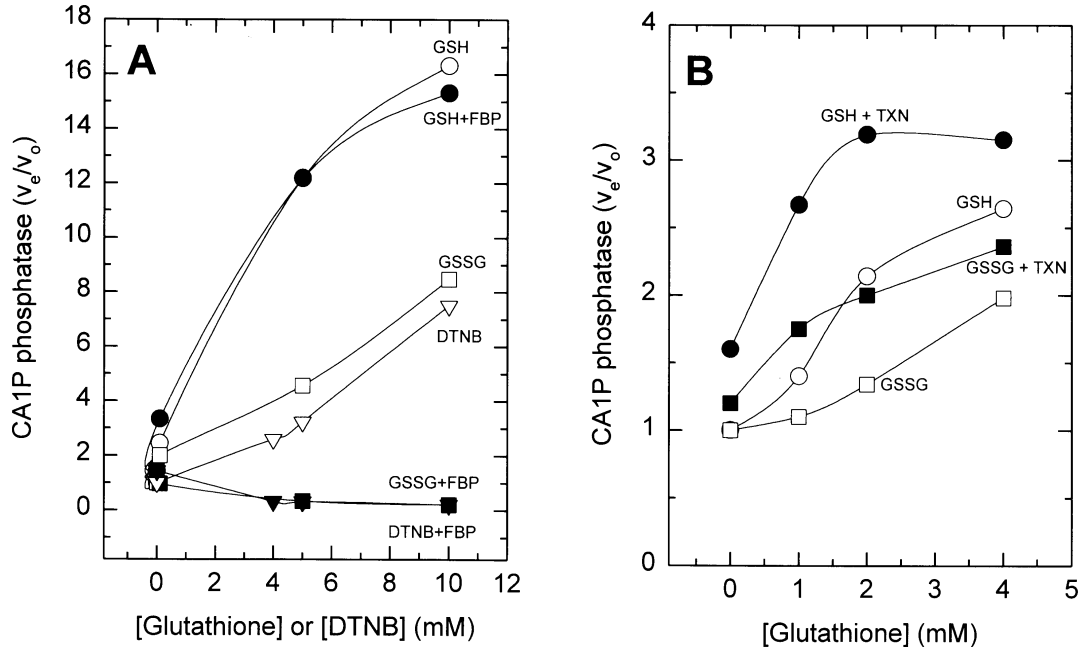


Figure 4 Interactive effects of FBP and *E. coli* thioredoxin on CA1P phosphatase in the presence of glutathione

(**A**) Green A column-purified enzyme was dialysed without DTT as described in Figure 1, and then incubated with GSH, GSSG or DTNB at the concentrations shown for 30 min. Assays were initiated after a further 5 min incubation with or without 5 mM FBP. (**B**) CA1P phosphatase from the Mono Q column step was dialysed with DTT for 12 h, and then incubated with GSH or GSSG for 30 min. Assays were initiated after a further 5 min incubation with or without 2.5 μM reduced *E. coli* thioredoxin (TXN). Control activities (v_0) were 23 nmol and 1.3 μmol of carboxy-D-arabinitol formed/min per mg of protein for experiments shown in (**A**) and (**B**) respectively. Control experiments established a close similarity in the responses of Green A column- and Mono Q column-purified CA1P phosphatase to glutathione, DTNB and FBP.

Table 2 Effect of light and darkness on the levels and redox state of homoglutathione in leaves of *P. vulgaris*

The homoglutathione levels were calculated by measurement of glutathione [25] and applying the correction factor ($\times 2.67$) for the greater reactivity of the hGSSG in the assay with glutathione reductase [26]. The concentration in chloroplasts was calculated assuming that 65% of cellular homoglutathione is in these plastids [40], and a stromal volume of 20.5 μl/mg of chlorophyll [41]. Total chloroplast sulphur content present as homoglutathione was corrected for hGSSG containing twice as much sulphur as hGSH.

	Light	Dark
Homoglutathione (nmol/mg of chlorophyll)	115.00	96.00
Estimated concentration in chloroplasts (mM)	3.66	3.06
% hGSH	94.80	70.20
% hGSSG	5.20	29.80
Total chloroplast S content present as homoglutathione (mM)	3.85	3.97
% hGSH based on total S content	90.00	54.00

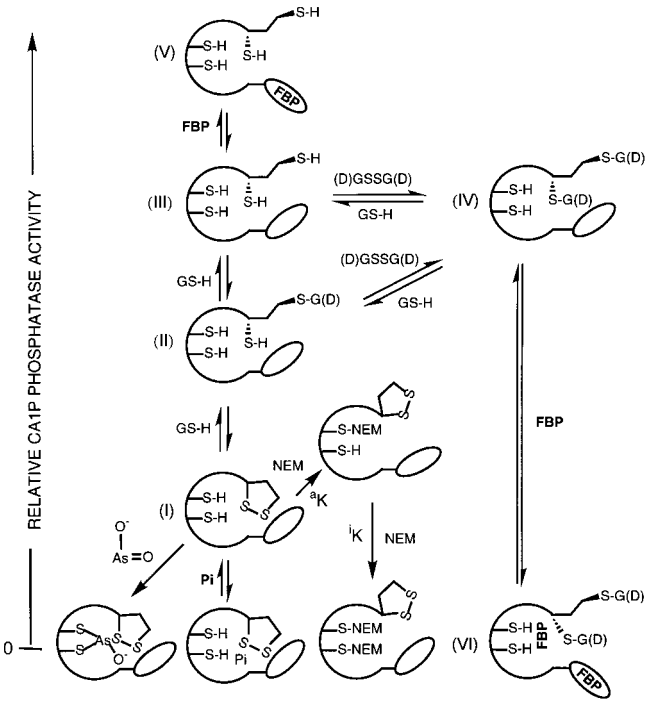
chloroplast (approx. 4 mM). If the percentage of hGSH present is based on total sulphur content, the effect of illumination appears more pronounced, causing a 36% increase in the level of hGSH. The measured increase in the proportion of hGSH in the light coincides very closely with the range in the percentage of GSH critical for modulating CA1P phosphatase activity *in vitro* (Figure 3C).

DISCUSSION

The results presented indicate that CA1P phosphatase activity is susceptible to redox regulation *in vitro*, which may be of importance to light regulation of this enzyme in the leaf. In previous work [10,18], and also in this study, DTT elicited a minor but significant stimulatory effect on CA1P phosphatase partially inactivated by air oxidation (Table 1). Furthermore, reduced *E. coli* thioredoxin induced greater enzyme activity in a shorter period of time than DTT alone. However, interactions of CA1P phosphatase with the low-molecular-mass monothiol, glutathione, were much more significant, as it enhanced catalytic rates by approximately 35-fold. In addition, CA1P phosphatase purified in the presence of DTT was five-fold less sensitive to activation by GSH than enzyme dialysed without DTT (Figure 4). This observation is consistent with a loss of activity due to air oxidation of critical enzyme thiols to form protein disulphides. Catalytic rates can then be stimulated beyond control rates by incubation with reduced thioredoxin or glutathione, reversing inhibition caused by oxidation. In contrast, data shown in Figures 1 and 2 established that CA1P phosphatase can be progressively inactivated by a range of thiol-modifying reagents. This also strongly indicates that the enzyme has specific thiols that are involved in regulatory or catalytic functions. Since the oxidizing agents GSSG and DTNB caused substantial activation of air-oxidized CA1P phosphatase, formation of mixed disulphides can also be considered as a mechanism resulting in an active form of the enzyme (Figures 1, 3 and 4). This outcome is notably different from the inhibitory effect of mixed disulphide formation on other redox-regulated enzymes [19,31].

Evidence that CA1P phosphatase has reactive external disulphides and internal vicinal thiols

Although inactivation of bean CA1P phosphatase as a result of dialysis in the absence of DTT was substantial, some residual activity was routinely observed. This partial inactivation of



Scheme 1 A proposed model for the redox regulation of CA1P phosphatase by glutathione and thiol reagents, and its possible interaction with FBP and P_i

Two discrete sets of thiol groups are shown, one internal and one external. The enzyme has minimal activity in the form of an intramolecular mixed disulphide of the external disulphide (form **I**). Intermediate activity occurs with intermediate mixed disulphides (form **II**), and full activity with the open form of both sets of thiol groups (form **III**) and a mixed disulphide (form **IV**). The internal thiol groups are resistant to air oxidation, and may be critical to catalysis. P_i may inhibit the enzyme by blocking the catalytic site directly. Form **III** can be activated by FBP additively, to give form **V**, whereas the exposed active site with the external mixed disulphide (form **IV**) is inhibited by FBP (form **VI**). Partial alkylation of the internal thiols by NEM may induce partial activation (^{a}K), and further alkylation inactivates the enzyme (^{i}K). Arsenite completely inhibits enzyme activity.

CA1P phosphatase by simple air oxidation over extended periods, and subsequent re-activation in the presence of GSH, GSSG and DTNB, indicates that a redox-active dithiol participating in the regulation of the enzyme is very solvent-accessible. In addition, the reagents, arsenite, *p*CMB, NEM and iodoacetate, which are specific for thiol groups [39], are capable of fully inhibiting the residual activity of the air-oxidized enzyme (Figure 1). It is therefore plausible that other thiol residues remain as thiol groups which are less accessible to air oxidation during dialysis of the enzyme, and so are most likely to be located in an internal region of the protein. The effectiveness of arsenite in blocking residual catalysis (Figure 1), and also in inhibiting activation by GSH (Figure 3), suggests that the internal thiols are vicinal.

A model for the regulation of CA1P phosphatase

Based on the evidence discussed above, we propose a possible model for the regulation of CA1P phosphatase by glutathione (Scheme 1). This representation of events also accounts for the additional influence of the effectors FBP and P_i, plus the interaction of the enzyme with the thiol reagents arsenite and NEM. In Scheme 1, form **I** represents air-oxidized CA1P phosphatase and shows two distinct thiol groups: an external oxidized intramolecular disulphide, and intact thiol groups at a site on the interior of the protein. Form **I** can be activated by either GSH or GSSG. We suggest that open forms of the external enzyme disulphide, forms **III** and **IV**, facilitate full activation of the enzyme. The external enzyme disulphide of form **I** can be converted to a fully active conformation (form **III**) by GSH or reduced cysteine. Alternatively, GSSG or DTNB can convert the external enzyme disulphide of form **I** to another opened mixed-enzyme disulphide form, form **IV**. However, the direct conversion from form **I** to form **IV** would be thermodynamically less favourable than the conversion of form **I** to form **III**. Also, considering data presented in Figure 4(A), the enzyme mixed disulphide (form **IV**) could expose the internal active site, creating a situation where FBP acts as an inhibitor (form **VI**, Scheme 1).

A physiological role for glutathione in the regulation of CA1P phosphatase?

The sigmoidal increase in CA1P phosphatase activity observed upon raising the percentage of GSH at relatively low fixed concentrations of total glutathione (Figure 3C) makes it feasible that this tripeptide could have a regulatory effect on the enzyme *in vivo*. Estimated stromal glutathione levels in chloroplasts of various plant species range from 3.5 to 20 mM [19], and are similar to the equivalent concentrations of homoglutathione measured for *P. vulgaris* (approx. 4 mM, Table 2). In addition, the proportion of hGSH was higher in illuminated leaves than in darkened leaves within a percentage range (54–90 %) consistent with the region of greatest redox sensitivity for CA1P phosphatase (Table 2, Figure 3C). The strong inhibition of catalysis by FBP in the presence of GSSG (Figure 4A, Scheme 1) may also enhance suppression of CA1P phosphatase activity by minor increases in the oxidized form of glutathione when leaves are darkened (Table 2; [40]).

The appreciable stimulation of CA1P phosphatase activity by GSH is unusual in view of the relatively positive reduction potential (-0.26 V) of glutathione as a monothiol. In comparison, DTT and thioredoxins f or m, which are all dithiols associated with substantial stimulation of certain other light-activated chloroplast enzymes [19,34], have reduction potential values that are distinctly more negative (-0.38 and -0.35 V respectively). Furthermore, the additional activation of CA1P phosphatase by GSSG indicates that this enzyme has a propensity for stimulation by mixed disulphide formation. Since the redox state of added glutathione also affects enzyme activity, the overall activation reflects a complex interaction between the total glutathione concentration and the [GSH]/[GSSG] ratio. Although the equilibrium oxidation potential [31] of CA1P phosphatase was not determined directly in this study, the data in Figure 3(C) indicate that half maximal activation of the enzyme is achieved at a [GSH]/[GSSG] ratio of approximately 2.0.

Referring again to Scheme 1, enhancement of CA1P phosphatase activity by each form of glutathione (Figure 3B) suggests that the conversion of form **I** to form **III** was favoured over the conversion of form **I** to form **IV** of the enzyme, over the range of 4–10 mM total sulphur content. However, at a higher concentration of sulphur (e.g. 15 mM) both putative conversions (form **I** to forms **III** and **IV**) occurred at the same rate as reflected by the extent of enzyme activation (Figure 3B).

Further investigations will examine the contribution of glutathione to the control of CA1P metabolism *in vivo*, and consequent effects on the light/dark regulation of Rubisco activity in leaves.

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