An affinity label for the regulatory dithiol of ribulose-5-phosphate kinase from maize (*Zea mays*)

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(Received 17 May 1983/Accepted 31 August 1983)

1. Ribulose-5-phosphate kinase from maize (*Zea mays*) can exist in either a reduced, active form or an oxidized, inactive form. Reduced ribulose-5-phosphate kinase is rapidly and irreversibly inactivated by the dichlorotriazine dye Reactive Red 1 (Procion Red MX-2B), but the irreversible inactivation of the oxidized form of ribulose-5-phosphate kinase occurs at only 0.05% of this rate. 2. The rate of inactivation of the reduced enzyme by Reactive Red 1 (apparent bimolecular rate constant $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.4 and 25°C) is several orders of magnitude greater than previous estimates of the rates of dye-mediated inactivation of other enzymes. 3. The dye-dependent inactivation of the reduced enzyme is inhibited by Hg^{2+} or *p*-mercuribenzoate (thiol reagents that reversibly inhibit ribulose-5-phosphate kinase activity), or by ATP and ADP, the nucleotide substrates of the enzyme. 4. Hydrolysed Reactive Red 1, which does not inactivate the enzyme, is a reversible inhibitor of ribulose-5-phosphate kinase. This inhibition is competitive with respect to ATP ($K_i \sim 0.5 \text{ mM}$). 5. The dye appears to act as an affinity label for the ATP/ADP-binding site by preferentially arylating a thiol residue generated during the reductive activation of the enzyme that is achieved by dithiothreitol or thioredoxin *in vitro* or during illumination of leaves.

Ribulose-5-phosphate kinase catalyses the synthesis of ribulose 1,5-bisphosphate, the CO_2 acceptor in the photosynthetic carbon-reduction cycle. The enzyme is inactive in darkened leaves and is activated when leaves or isolated chloroplasts are illuminated (Latzko *et al.*, 1970; Steiger *et al.*, 1971; Laing *et al.*, 1981). The isolated, oxidized enzyme can be activated by incubation of the enzyme with dithiothreitol or reduced thioredoxin (Latzko *et al.*, 1970; Wolosiuk & Buchanan, 1978) and conversely the active, reduced enzyme can be inactivated by oxidizing agents (Wolosiuk & Buchanan, 1978; Ashton, 1983). The oxidized enzyme from maize (*Zea mays*) has no detectable activity over a wide pH range (Ashton, 1983). Thus synthesis of ribulose 1,5-bisphosphate and consequently functioning of the photosynthetic carbon-reduction cycle is absolutely dependent upon the reduction of ribulose-5-phosphate kinase. It appears that the dithiol/disulphide group that undergoes reversible oxidation/reduction is critical to the light/dark-dependent regulation of the enzyme. This dithiol/disulphide may occur near the aden-

ine-nucleotide-binding site of the enzyme, since ATP and ADP inhibit both the reductive activation and the oxidative inactivation of the enzyme (Ashton, 1983; A. R. Ashton, unpublished work). This reductive light-activation process is also a mechanism for regulating the activity of a number of other enzymes of photosynthetic carbon metabolism, including NADP:malate dehydrogenase, fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase [for reviews, see Anderson *et al.* (1982) and Buchanan (1980)].

The present study describes the interaction of the reactive dye Procion Red MX-2B (Reactive Red 1; Fig. 1) with reduced and oxidized ribulose-5-phosphate kinase from *Zea mays*. Various dyes containing the reactive chlorotriazine group can function as affinity labels that react with amino acid residues near the dye-binding site, which frequently is the enzyme active site (Clonis & Lowe, 1980; Witt & Roskowski, 1980). Furthermore, in the case of NADP:malate dehydrogenase from *Z. mays*, dichlorotriazine dyes, particularly Procion Rubine MX-B (Reactive Red 6) apparently react rapidly and specifically with the regulatory dithiol of the enzyme (A. R. Ashton, unpublished work). The present paper reports that Reactive Red 1, which is a competitive inhibitor of ribulose-5-

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Fig. 1. Structure of Procion Red MX-2B (Reactive Red 1; C.I. 18158)

$R_1 = R_2 = \text{Cl}$, Reactive Red 1; $R_1 = R_2 = \text{OH}$, hydrolysed Reactive Red 1; R_1 or $R_2 = S\text{-cysteinyl-enzyme}$, proposed structure of inactivated ribulose-5-phosphate kinase.

phosphate kinase, very rapidly and specifically inactivates ribulose-5-phosphate kinase, apparently by arylating the regulatory dithiol of the enzyme. Evidence is presented that this dithiol is located at or near the adenine-nucleotide-binding site of the enzyme.

Experimental

Materials

Bovine serum albumin (fatty-acid-poor) and ribose-5-phosphate isomerase were obtained from Calbiochem, whereas other biochemicals were obtained from Sigma. Procion Brilliant Red MX-2B (Reactive Red 1 C.I. 18158) and other Procion dyes were provided by Mr. K. Brown of ICI (Australia). Reactive Red 1 was hydrolysed by heating the dye in Na_2CO_3 solution (10mg/ml) for 6h at 70°C . Hydrolysis of the dye was confirmed by cellulose t.l.c. [solvents: butanol/ethanol/water, 10:7:8 (by vol.) and pyridine/butanol/water, 1:1:1 (by vol.)] of the hydrolysed dye.

Ribulose-5-phosphate kinase was partially purified from leaves of *Zea mays* in 50mM-Tris/HCl/10mM-KCl/1mM-EDTA/2mM- MgCl_2 , pH 7.4 (Tris/KCl buffer) at $0\text{--}4^\circ\text{C}$ as summarized below. The Tris/KCl buffer used for homogenization contained 50mM-2-mercaptoethanol and insoluble polyvinylpyrrolidone (10%, w/w, with respect to leaves). All subsequent Tris/KCl buffer solutions did not contain any reducing agent. The purification procedure involved sequential $(\text{NH}_4)_2\text{SO}_4$ fractionation (40–75% satn.), poly(ethylene glycol) 6000 fractionation (15–25%, w/v), DEAE-cellulose (10–400mM-KCl gradient) and Sephacryl S-300 chromatography. The final specific activity of the enzyme was $58\mu\text{mol}$ of ATP utilized/min per mg of protein, with an overall yield of 20%. The ATPase activity of the enzyme in

Fig. 2. Time course of inactivation of reduced ribulose-5-phosphate kinase by Reactive Red 1

The time course of enzyme inactivation by Reactive Red 1 (or its hydrolysis product) in Tris/KCl buffer was conducted as described in the Experimental section. (a) $\circ$, $10\mu\text{M}$ -Reactive Red 1; $\bullet$, reaction mixture containing enzyme and 2.5mg of bovine serum albumin/ml, started by addition of $10\mu\text{M}$ -Reactive Red 1; $\square$, a reaction mixture containing 50mM-dithiothreitol and $10\mu\text{M}$ -Reactive Red 1 preincubated for 5 min (25°C) before addition of enzyme; $\triangle$, $10\mu\text{M}$ -hydrolysed Reactive Red 1. (b) The inactivation reaction contained $1\mu\text{M}$ -Reactive Red 1, alone ($\circ$) or in the presence of 0.2mM-mercuric acetate ($\square$) or 0.1mM-*p*-mercuribenzoate ($\triangle$).

the absence of ribulose 5-phosphate was less than 0.1% of ribulose-5-phosphate kinase activity. The oxidized, inactive enzyme (in Tris/KCl buffer) was activated by incubation for 16h at 20°C with 100mM-dithiothreitol. Before use, the active en-

Fig. 3. Time course of inactivation of reduced ribulose-5-phosphate kinase by *N*-ethylmaleimide and Reactive Red 1 in the presence or absence of dithiothreitol

The time course of inactivation of reduced ribulose-5-phosphate kinase was conducted as described in the Experimental section. The inactivation reaction was started by addition of *N*-ethylmaleimide or Reactive Red 1. *N*-ethylmaleimide (5 μ M) with (■) or without (□) 1 mM-dithiothreitol; Reactive Red 1 (1 μ M) with (●) or without (○) 1 mM-dithiothreitol.

zyme was freed of dithiothreitol by gel filtration through a Sephadex G-25 column equilibrated with 1 mM-EDTA, pH 6.0, and then stored at 4°C under an N_2 atmosphere.

Inactivation of ribulose-5-phosphate kinase

The 250 μ l reaction mixtures contained 50 mM-Tris/HCl, 10 mM-KCl, 2 mM-MgCl₂ and 1 mM-EDTA, pH 7.4, and were incubated at 25°C. At intervals, portions (25 μ l) were removed and the inactivation reaction terminated by dilution into enzyme assay mixture (1 ml) containing 2.5 mg of bovine serum albumin/ml and 10 mM-dithiothreitol. The albumin in the assay mixture binds the free dye, which otherwise might inhibit the enzyme by binding reversibly to the active site. The dithiothreitol reacts with *N*-ethylmaleimide carried into the assay and relieves the inhibition of the enzyme by mercurials.

Enzyme assay

Ribulose-5-phosphate kinase activity was measured at 25°C in a 1 ml reaction mixture containing 25 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, 1 mM-EDTA, 25 mM-KCl, 10 mM-MgSO₄, 1 mM-ATP, 2 mM-phosphoenolpyruvate, 0.2 mM-NADH and 2 mM-

ribose-5-phosphate, adjusted to pH 8.3 with KOH. Ribose-5-phosphate isomerase (1 unit), pyruvate kinase (3 units) and lactate dehydrogenase (5 units) were present as coupling enzymes.

Results and discussion

Ribulose-5-phosphate kinase, purified from *Zea mays*, in the absence of reducing agents is completely inactive, but can be activated by incubation with thiols, including dithiothreitol (Ashton, 1983). Reduced, active ribulose-5-phosphate kinase is rapidly inactivated by 1 μ M-Reactive Red 1 (Fig. 2). The rate of enzyme inactivation by other dyes was considerably less than that for Reactive Red 1. For example Reactive Red 6, Reactive Blue 4, Reactive Yellow 4, Reactive Red 8 and Reactive Blue 2 inactivated the reduced enzyme at 20, 12, 2, 1 and <0.5% respectively of the rate at which Reactive Red 1 inactivated the enzyme. This inactivation is apparently irreversible, since it is not reversed by prolonged incubation of the inactivated enzyme with dithiothreitol, which would reactivate any oxidized enzyme, and bovine serum albumin, which binds most of the free dye in the reaction mixture. The presence of serum albumin in an inactivation reaction prevents inactivation of the enzyme (Fig. 2a), presumably by sequestering most of the reactive dye. Hydrolysis of the reactive chlorine groups on the dye (Fig. 1) largely prevents enzyme inactivation, suggesting that inactivation results from arylation of an enzyme amino acid residue (Fig. 2). The rate of inactivation of the enzyme by Reactive Red 1 deviates from simple pseudo-first-order kinetics. Similar deviations have been previously reported to occur in the Reactive-Dye-dependent inactivations of other enzymes (Clonis & Lowe, 1980) and were attributed to dye hydrolysis or changes in the degree of dye polymerization. It may also be possible that the micromolar concentrations of Reactive Red 1 used here may be significantly adsorbed to the sides of the test tube containing the reaction mixtures. This is consistent with the observation that very dilute solutions of Reactive Red 1 (10 μ M) lose their inhibitory activity quickly, whereas more concentrated solutions (1 mM) are stable indefinitely. All results reported here were conducted with dye freshly diluted from such concentrated stock solutions. Clonis & Lowe (1980) and Witt & Roskowski (1980) have presented evidence that other reactive chlorotriazine dyes act as affinity labels for dye-binding sites on various enzymes by initially binding reversibly to the enzyme (E-dye) and when bound occasionally reacting with an amino acid residue at the dye binding site as follows (Plapp, 1982).

This reaction sequence requires that the rate of enzyme inactivation yields hyperbolic saturation kinetics as the dye concentration increases. The initial rate of inactivation of ribulose-5-phosphate kinase at low concentrations of Reactive Red 1 is proportional to dye concentration (t_i for inactivation by $1 \mu\text{M}$ -dye ~ 1 min, t_i for $10 \mu\text{M}$ -dye ~ 0.1 min), but at higher dye concentrations the inactivation proceeds too rapidly to be measured reliably. As discussed by Plapp (1982), saturation kinetics may not be observed if k_{-1} and k_{+2} are relatively high and therefore the rate of enzyme inactivation at dye concentrations approaching K_d is too swift to measure without sophisticated methods. Nevertheless the apparent bimolecular rate constant ($0.693/t_i [\text{dye}]$ or k_{+2}/K_d) for the inactivation of ribulose-5-phosphate kinase by Reactive Red 1 can be estimated to be $\sim 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (i.e. t_i for inactivation of 1 min at $1 \mu\text{M}$ -dye). Further-

Fig. 4. Time course of inactivation of oxidized and reduced ribulose-5-phosphate kinase by Reactive Red 1

The inactivation of oxidized (●) and reduced (○) ribulose-5-phosphate kinase (by $100 \mu\text{M}$ - and $10 \mu\text{M}$ -Reactive Red 1 respectively) was conducted as described in the Experimental section. The oxidized enzyme was activated before assay by incubating a $25 \mu\text{l}$ portion from the inactivation reaction with 100 mM -dithiothreitol, 0.25% bovine serum albumin, 25 mM -Tricine, 1 mM -EDTA, pH 8.3, in a final volume of $100 \mu\text{l}$, for 180 min at 25°C .

more, evidence presented below supports the proposal that Reactive Red 1 acts as an affinity label when it inactivates ribulose-5-phosphate kinase.

Ribulose-5-phosphate kinase is inhibited by the thiol reagents mercuric ions and *p*-mercuribenzoate and this inhibition is rapidly reversed by excess dithiothreitol (A. R. Ashton, unpublished work). Moreover, the inactivation of the enzyme by Reactive Red 1 is prevented by mercuric acetate and *p*-mercuribenzoate (Fig. 2b). The dye reacts with thiol groups, since preincubation of the dye with dithiothreitol prevents enzyme inactivation (Fig. 2a). Apparently the dye inactivates the enzyme by arylating an enzyme thiol residue (Fig. 1), but does not react when the thiol is blocked by a mercurial. Recently the affinity labelling of horse liver alcohol dehydrogenase by Procion Blue MX-R (Reactive Blue 4) was shown to occur by modification of a specific cysteine residue within the catalytic domain (Small *et al.*, 1982). Reactive Red 1 apparently reacts far more rapidly with the enzyme thiol than with low- M_r thiols, since $1 \mu\text{M}$ -Reactive Red 1 still rapidly inactivates the enzyme in the presence of 1 mM -dithiothreitol (Fig. 3). This effectiveness of the enzyme thiol in competing with dithiothreitol for the Reactive Red 1 cannot be attributed to hyper-reactivity of the enzyme thiol, since $5 \mu\text{M}$ -*N*-ethylmaleimide, which also rapidly inactivates ribulose-5-phosphate kinase apparently by reacting with the same thiol (see below), does not inhibit the enzyme in the presence of 1 mM -dithiothreitol (Fig. 3). A plausible explanation for these observations is that the reaction between the dye and enzyme thiol is enhanced by a reversible preliminary binding of the dye to the enzyme near the thiol residue. By contrast with the reduced ribulose-5-phosphate kinase, the oxidized enzyme is inactivated by Reactive Red 1 at only 0.05% of the rate at which the reduced form of the enzyme is inactivated (Fig. 4). This low rate of inactivation of the oxidized enzyme may reflect reaction of the dye with other amino acid residues at the dye-binding site (Clonis & Lowe, 1980; A. R. Ashton, unpublished work). Similarly the oxidized enzyme is resistant to inactivation by *N*-ethylmaleimide (A. R. Ashton, unpublished work). This supports the proposal that the reduced enzyme is inactivated by Reactive Red 1 by arylation of the regulatory thiol that is generated upon reduction of the oxidized enzyme.

Since the high-affinity dye-binding site on many enzymes is apparently the nucleotide-binding site at the enzyme active site (Stellwagen, 1977; Ashton & Polya, 1978; Beissner & Rudolph, 1978), dyes that inactivate a number of enzymes frequently act as active-site-directed affinity labels (Witt & Roskowski, 1980; Clonis & Lowe, 1980). The inactivation of ribulose-5-phosphate kinase by

Fig. 5. Time course of inactivation of reduced ribulose-5-phosphate kinase by Reactive Red 1 in the presence of adenine nucleotides

The time course of inactivation of reduced ribulose-5-phosphate kinase by $1\mu\text{M}$ -Reactive Red 1 was conducted as described in the Experimental section. Reactive Red 1 ($1\mu\text{M}$) alone ($\circ$) or in the presence of 1mM -ATP (Δ) or 1mM -ADP ($\square$).

Fig. 6. Time course of inactivation of reduced ribulose-5-phosphate kinase by *N*-ethylmaleimide in the presence of adenine nucleotides

The time course of inactivation of reduced enzyme by *N*-ethylmaleimide was conducted as described in the Experimental section. The reaction mixtures contained $5\mu\text{M}$ -*N*-ethylmaleimide, alone ($\circ$) or in the presence of 5mM -ATP (Δ) or 5mM -ADP ($\square$).

Reactive Red 1 is prevented by ATP or ADP, the nucleotide substrates of the enzyme (Fig. 5), suggesting that the arylated amino acid residue is at the ATP/ADP-binding site. Moreover, the *N*-ethylmaleimide-dependent inactivation of the enzyme (Fig. 6), which is also prevented by these nucleotide substrates, indicates the presence of a reactive thiol residue at the adenine-nucleotide-binding site. Hydrolysed Reactive Red 1 reversibly inhibits ribulose-5-phosphate kinase and this inhibition is competitive with respect to ATP (Fig. 7), further indicating that Reactive Red 1 interacts at the ATP/ADP-binding site of the enzyme. The extent of enzyme inhibition by hydrolysed Reactive Red 1 does not increase with time, suggesting that the enzyme is not being inactivated in the assay, nor is the dye a slow-binding inhibitor of ribulose-5-phosphate kinase, whereas a number of dyes are slow-binding inhibitors of NADP: malate dehydrogenase (A. R. Ashton, unpublished work). The K_i of ribulose-5-phosphate kinase for hydrolysed Reactive Red 1 is $\sim 0.5\text{mM}$. The absence of saturation kinetics for the inactivation of ribulose-5-phosphate kinase could be explained if the K_d for the dichlorotriazine dye is also $\sim 0.5\text{mM}$.

The evidence presented here suggests that Reactive Red 1 inactivates reduced ribulose-5-phosphate kinase by arylating an enzyme thiol generated during the reductive activation of the enzyme. Furthermore, this thiol residue is appar-

Fig. 7. Competitive inhibition of ribulose-5-phosphate kinase by hydrolysed Reactive Red 1

The activity of ribulose-5-phosphate kinase in the routine assay mixture containing 1mM -dithiothreitol was determined at various ATP concentrations in the absence ($\circ$) or presence of hydrolysed Reactive Red 1 ($\bullet$, 0.52mM ; $\square$, 0.26mM). The coupling enzymes were still active in the presence of these concentrations of dye. The lines, drawn by eye, intersect at $1/v = 0.54$ unit.

ently located at or near the adenine-nucleotide-binding site of the enzyme. Additional support for this view is provided by the observations that ATP and ADP inhibit both the reductive activation and the oxidative inactivation of ribulose-5-phosphate kinase (Ashton, 1983; A. R. Ashton, unpublished work).

Reactive Red 1 inactivates reduced ribulose-5-phosphate kinase approx. 20 times faster than the rate of Reactive Red 6-dependent inactivation of reduced NADP:malate dehydrogenase (A. R. Ashton, unpublished work), which in turn was inactivated 100-fold faster than lactate dehydrogenase, hexokinase and glucose-6-phosphate dehydrogenase were inactivated by a variety of dichlorotriazine dyes (Clonis & Lowe, 1980). It appears that the regulatory thiol residues of these two light-activated enzymes are particularly susceptible to arylation by some, but not all, dichlorotriazine dyes. Presumably the more effective dyes bind to the enzyme such that the dichlorotriazine ring is oriented correctly toward the regulatory thiol. Since a wide variety of structurally dissimilar dyes bind to enzyme active sites (Ashton & Polya, 1978), it seems unlikely that any one part of the dye, for example the triazine ring, always binds in the same position within the active site. By screening a variety of dyes it should prove possible to obtain more potent and selective enzyme inhibitors as has been done here for ribulose-5-phosphate kinase and previously for NADP:malate dehydrogenase (A. R. Ashton, unpublished work).

The reaction of Reactive Red 1 with reduced ribulose-5-phosphate kinase may provide a means of identifying the thiol residues involved in the

light-dependent activation of this enzyme. This reactive dye or others may also provide a means for inhibiting specific light-activated enzymes of the chloroplast in reconstituted systems or *in vivo*.

This work was supported by a Queen Elizabeth II Fellowship awarded to me and was conducted in the laboratory of Dr. M. D. Hatch.

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