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Photomodification of a Serine at the Active Site of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase by Vanadate†

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Received January 10, 1989; Revised Manuscript Received March 14, 1989

ABSTRACT: Irradiation of ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach in the presence of vanadate at 4 °C resulted in rapid loss of carboxylase activity. The inactivation was light and vanadate dependent. When the enzyme was irradiated in the presence of the substrate ribulose 1,5-bisphosphate or an analogue such as fructose 1,6-bisphosphate, the inactivation was greatly reduced. Sodium bicarbonate and phosphate also protected against inactivation. No additional protection was observed in the presence of Mg^{2+} nor did Mg^{2+} alone protect. Carboxylase activity could be partially restored by treatment with $NaBH_4$, and the photomodified protein could be tritiated with NaB^3H_4. Amino acid analysis showed that the tritium had been incorporated into serine. The data suggest that an active-site serine is photooxidized by vanadate to an aldehyde which results in activity loss. Irradiation in the presence of vanadate also resulted in cleavage in the large subunit of the enzyme which was subsequent to inactivation.

The enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[1] catalyzes the carboxylation of ribulose 1,5-bisphosphate (RuBP). This is the initial step in carbon fixation [for a review, see Miziorko and Lorimer (1983)]. The enzyme also catalyzes the oxygenation of RuBP, which leads to photorespiration (Andrews & Lorimer, 1978), resulting in a net loss of carbon.

The enzyme in higher plants is a hexadecamer consisting of eight large 55-kDa subunits and eight small 15-kDa subunits. The active site is located on the large subunit, while the role of the small subunit is unknown. In light of the importance of RuBisCO to virtually all life, the active site of the enzyme has been extensively studied. Various affinity labels and protein modification reagents have suggested that two lysines, a cysteine, histidine, tyrosine, arginine, methionine, and a glutamyl or aspartyl residue (Valle & Vallejos, 1974) are in the active-site domain (for a review, see McFadden and Small 1988). However, there have been no reports implicating serine. A serine has been identified in the active site of skeletal myosin using orthovanadate (V_i) trapped at the active site by ADP and Mg^{2+} (Cremo et al., 1988). Upon irradiation of the myosin·V_i·ADP·Mg^{2+} complex, myosin is ultimately modified twice. The first modification results in the oxidation of an active-site serine to the corresponding aldehyde. If V_i is then retrapped at the active site and the complex is exposed to UV light, a second modification occurs resulting in cleavage of the peptide backbone, apparently at the oxidized serine (Grammer et al., 1988).

We now present evidence to support UV-induced, V_i-dependent oxidation of a serine at the active site of spinach RuBisCO to an aldehyde which results in activity loss, and subsequent cleavage of the enzyme.

Experimental Procedures

Materials. Sodium salts of RuBP and FBP were purchased from Sigma Chemical Co. Orthovanadate (V_i) was prepared from V_2O_5 as described by Goodno (1982). Spinach RuBisCO was prepared from fresh spinach leaves (Berhow et al., 1982) and stored at −70 °C in the nonactivated state. NaB^3H_4 and $NaH^{14}CO_3$ were obtained from Dupont–NEN and ICN, respectively. CABP was synthesized as described by Pierce et al. (1980).

Photomodification. Unless otherwise indicated, irradiation was performed in the presence of 0.3 mM V_i in 50 mM MOPS buffer at pH 7.5. The protein concentration was 0.3 mg/mL (4.3 μM active sites) at the time of irradiation, which was performed on ice with a Hanovia 450-W medium-pressure Hg lamp (Ace Glass) at a distance of 9 cm. A glass filter was used to prevent surface heating and to screen out radiation below 330 nm. The samples were kept on ice during irradiation. The initial rate of inactivation was determined by

† This research was supported in part by NIH Grant GM-19972.
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[1] Abbreviations: CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; DTT, DL-dithiothreitol; FBP, D-fructose 1,6-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P_i, orthophosphate; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SDS, sodium dodecyl sulfate; UV, ultraviolet; V_i, orthovanadate ($H_2VO_4^-$).

0006-2960/89/0428-5428$01.50/0

measuring the RuBP carboxylase activities of samples of RuBisCO irradiated in the presence of indicated concentrations of V_i for 1.0, 3, and 5 min.

Reduction and RuBP Carboxylase Activity. After irradiation, an indicated amount of $NaBH_4$, prepared in 0.1 N NaOH, was added to the photomodified sample. The samples were incubated for 1 h at 25 °C. Fructose was then added to a final concentration of 23 mM to quench the remaining $NaBH_4$. The samples were allowed to react for an additional hour at 25 °C. The samples were then activated and assayed for RuBP carboxylase activity as described by McFadden et al. (1975). Activity (micromoles of RuBP-dependent CO_2 fixed per minute) is expressed as a percentage relative to a nonirradiated sample treated otherwise identically. Unmodified enzyme has a specific activity of 2.2 units min^{-1} (mg of protein)$^{-1}$ where 1 unit of enzyme catalyzes the RuBP-dependent fixation of 1 μmole of CO_2/min.

Tritiation. The alkaline solution of NaB^3H_4 was added to irradiated samples to a final concentration of 0.6 mM (specific activity = 156 mCi/mmol). The samples were allowed to react for 1 h at 25 °C. The reaction was quenched with an equal volume of 12 N HCl, and the samples were desiccated in vacuo (<0.1 mmHg) at room temperature to near-dryness to remove volatile tritium. To each sample was then added 0.5 mL of 0.1 N HCl and the desiccation repeated. The samples were resuspended in 4% SDS and subjected to centrifugation through a 5-mL column of Sephadex G-50 as described by Penefsky (1977). The samples were then counted for tritium in an aqueous counting scintillant (Amersham Corp.) using a Beckman LS9000 liquid scintillation counter.

Amino Acid Analysis. Tritiation of irradiated enzyme was performed as described above. The tritiated protein was hydrolyzed in 6 N HCl for 22 h in vacuo under N_2 at 110 °C. Analysis was performed with an LKB Alpha Plus amino acid analyzer after ion-exchange chromatography (Moore & Stein, 1963). Fractions were collected, and the ninhydrin color was allowed to fade for 14 h at 25 °C. The fractions were then counted for tritium as described above.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was performed essentially as described by Laemmli (1970). Ultrapure grade acrylamide and molecular weight standards were from Bio-Rad Laboratories.

Results

UV-Induced Inactivation of RuBisCO by Vanadate. RuBisCO was exposed to UV light in the presence of V_i and was rapidly inactivated. Inactivation was irreversible in that activity could not be restored by dialysis or gel filtration. The inactivation was vanadate dependent and exhibited saturation kinetics (Figure 1). In the presence of 0.3 mM V_i, RuBP carboxylase activity was lost with a half-life of 4.25 min of irradiation (Figure 2). By contrast, in the absence of vanadate, only a slight loss of activity was observed after 30 min of irradiation (Figure 2). The substrate RuBP and the inhibitor FBP (McFadden, 1973) both protected against inactivation (Figure 2). It is known that compounds with hydroxyl groups can form complexes with vanadate (Gresser & Tracey, 1985, 1986). However, this cannot explain the protection by these compounds since fructose gave no protection against inactivation (Figure 2). The presence of 10 mM phosphate also partially protected against inactivation (Figure 2).

The protection by bicarbonate shown in Figure 2 suggests that carbamylation of lysine-201 (Miziorko & Lorimer, 1983) may partially protect the enzyme against photoinactivation by V_i. Although nonsubstrate bicarbonate is known to bind

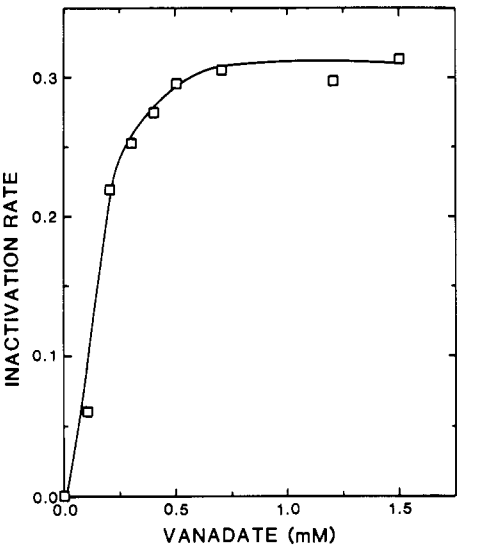

FIGURE 1: Dependence of the rate of photoinactivation at 4 °C on the concentration of V_i.

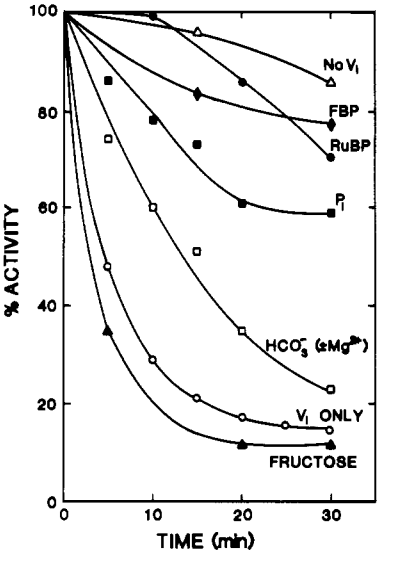

FIGURE 2: Photoinactivation of RuBP carboxylase activity as a function of time. Irradiation was performed in the presence of no vanadate (♦) or 0.3 mM vanadate (○) plus 1 mM RuBP (●), 1 mM FBP (♦), 10 mM $NaHCO_3$ (with or without $MgCl_2$) (□), 10 mM Na_2HPO_4 (■), or 1 mM fructose (▲). All samples were adjusted to pH 7.5 before irradiation.

competitively at or near the phosphate binding site of the activated enzyme (Pierce et al., 1986), analogous anions such as formate and acetate afforded no protection whatsoever (data not shown).

Restoration of Activity by $NaBH_4$. When RuBisCO was irradiated for 10 min in the presence of 0.3 mM V_i, approximately 16% of the initial carboxylase activity remained. The activity could be partially restored by treatment with sodium borohydride as shown in Figure 3. However, activity was not fully restored, and this may have been due, in part, to a second irreversible modification resulting in cleavage of the enzyme (see below). When $NaBH_4$ was removed by gel filtration before the carboxylase assays were performed, higher restoration of activity was not observed (data not shown).

Activity was not restored by other reducing agents such as DDT or 2-mercaptoethanol (data not shown).

Tritium Incorporation and Amino Acid Analysis. If indeed a serine had been oxidized to an aldehyde during irradiation in the presence of V_i, then the modified enzyme should have

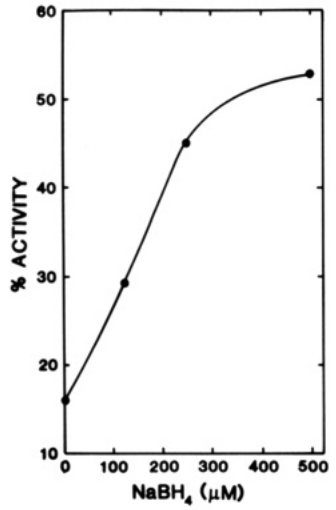

FIGURE 3: Restoration of carboxylase activity by $NaBH_4$. Irradiation was performed at 4 °C for 15 min. The activity of irradiated and reduced samples is plotted as a percentage relative to the activity of a nonirradiated sample treated otherwise identically.

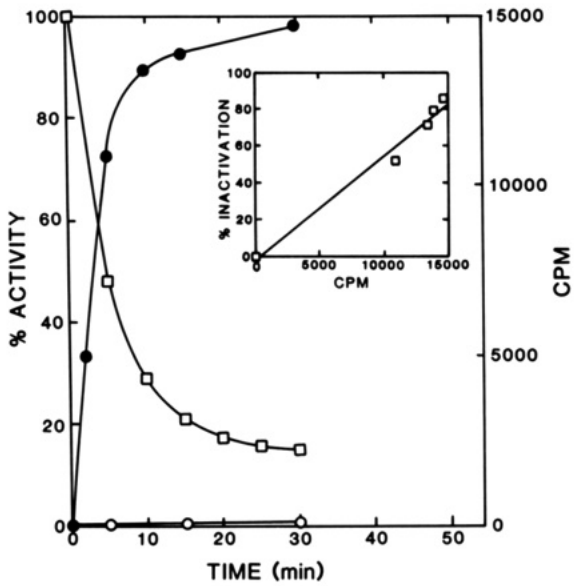

FIGURE 4: Tritium incorporation (cpm) by RuBisCO after photomodification in the absence (●) or presence of CABP (○) and activity loss of RuBisCO photomodified in the absence of CABP (□) as a function of irradiation time. After the indicated time of irradiation at 4 °C, samples containing 40 μg of RuBisCO were reacted with NaB^3H_4 as described under Experimental Procedures. The corresponding loss of carboxylase activity is also shown as a function of irradiation time. The inset displays the relationship between cpm incorporated and inactivation during photomodification in the absence of CABP. The correlation coefficient for the inset was 0.99.

been susceptible to tritium incorporation using NaB^3H_4. Moreover, the extent of tritium incorporation should have correlated with activity loss due to oxidation. As evident, both expectations were met (Figure 4). In addition, the tight binding competitive inhibitor CABP (Pierce et al., 1980) prevented subsequent tritium incorporation when present during irradiation (Figure 4). It had no effect when added after irradiation during treatment with NaB^3H_4. CABP was used because it does not react with $NaBH_4$.

The stoichiometry of carbonyl formation could not be determined due to the unknown magnitude of isotope discrimination in carbonyl reduction. For example, we tritiated several carbonyl compounds at equimolar concentrations including pyruvate, glucose 6-sulfate, glyoxylate, and 4-(dimethylamino)benzaldehyde with 1 mM NaB^3H_4 for 48 h at 25 °C.

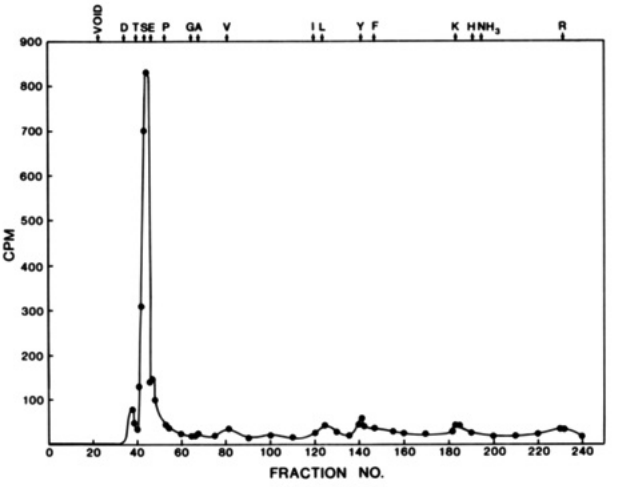

FIGURE 5: Amino acid analysis of 0.5 nmol of tritiated photomodified RuBisCO.

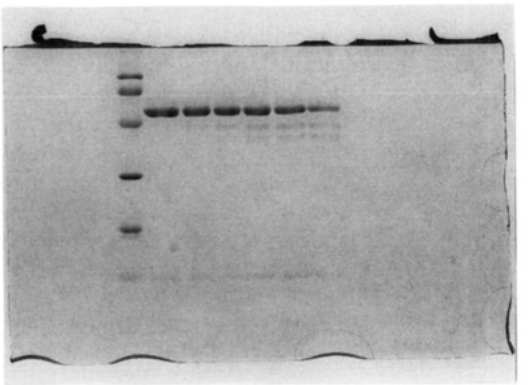

FIGURE 6: Vanadate-dependent photocleavage of RuBisCO shown by SDS–polyacrylamide gel electrophoresis. The gel was cast using a final concentration of 15% acrylamide and 0.4% *N,N'*-methylenebis(acrylamide). Electrophoresis was performed for 4.5 h at 150 V. The following standards were applied to the left lane: phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa) carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Applied to each other lane was 7.6 μg of RuBisCO which had been irradiated with 0.3 mM V_i for (left to right) 0, 5, 10, 20, 30, and 60 min.

Tritium incorporation varied as much as 5-fold (data not shown).

Either serine or threonine, if oxidized to an aldehyde or ketone, would incorporate tritium when reacted with NaB^3H_4. An analysis of the hydrolysate of RuBisCO after photomodification and reduction by NaB^3H_4 (Figure 5) showed that essentially all tritium is incorporated into serine.

Photocleavage. Irradiation of RuBisCO in the presence of V_i eventually led to cleavage of the polypeptide backbone (Figure 6). This also occurs with skeletal myosin (Grammer et al., 1988) as well as with dynein (Lee-Eiford et al., 1986). However, although 52% of the carboxylase activity was lost in 5 min of irradiation, only 2% of the large subunits were cleaved in that time. With longer irradiation times, cleavage became significant, resulting in at least five bands. No cleavage of the small subunits was apparent although fragments may have been overlooked under the conditions of electrophoresis employed (Figure 6). When 1 mM RuBP was present during irradiation, no cleavage was observed (data not shown).

DISCUSSION

Vanadate rapidly inactivates RuBisCO when irradiated with UV light. The inactivation by vanadate displays saturation

kinetics and substrate protection, indicating that an enzyme·V_i complex is formed and that modification resulting in loss of activity is probably occurring at the active site. Partial protection against photoinactivation by phosphate may suggest that vanadate is binding to the phosphate binding site(s). However, vanadate readily forms oligomers (Chasteen, 1983; Borgen et al., 1977). The oligomeric state of vanadate which binds to the enzyme and is responsible for photomodification of RuBisCO is currently under investigation.

Partial protection by bicarbonate may indicate that the carbamylated enzyme is more resistant to photoinactivation. Although nonsubstrate bicarbonate does bind to the active site and actually competitively inhibits RuBP binding (Pierce et al., 1986), anions such as formate and, perhaps, acetate would be expected to behave similarly. The lack of protection by these compounds suggests that the mode of bicarbonate protection may be due to carbamylation. Carbamylation may afford protection by inducing a conformational change at the active site, or the resultant negatively charged carbamyl moiety may simply repel negatively charged vanadate. The lack of any effect by Mg^{2+} suggests that it is the binding of CO_2 (or bicarbonate) that confers resistance to photomodification, and not the formation of the ternary complex, enzyme·CO_2·Mg^{2+}, which is required for activation of the enzyme.

The observation that carboxylase activity could be partially restored by $NaBH_4$ treatment suggested the generation of an enzymic carbonyl group during photoinactivation by vanadate. This has been confirmed by tritiation of the modified RuBisCO with NaB^3H_4. Oxidation of a serine to the corresponding aldehyde was postulated on the basis of similar results obtained during the photomodification of skeletal myosin in the presence of V_i (Cremo et al., 1988). In the present research, the inability of DTT to restore activity indicates that oxidation of cysteines to form a disulfide linkage is not responsible for activity loss. The recovery of tritiated serine in the amino acid hydrolysate of RuBisCO confirms that the borohydride-reducible group introduced by photomodification is a "serine aldehyde". Since reduction of the modified enzyme with unlabeled $NaBH_4$ partially restores activity, and the appearance of the borohydride-reducible group correlates with loss of activity, we conclude that the oxidation is responsible for most, if not all, of the activity loss. It may be relevant that serine-379 has very recently been placed in the active-site domain of spinach RuBisCO on the basis of crystallography (Andersson et al., 1989).

The reactions catalyzed by myosin ATPase and RuBP carboxylase have little in common. While the former involves cleavage of a phospho–anhydride bond, the latter does not involve alteration of the phosphate moiety. Nevertheless, both enzymes must bind phosphate-containing compounds. The observation that both enzymes seem to be photomodified by vanadate in similar ways suggests that vanadate may be useful as a general probe for the active site of phosphate binding enzymes. The results with myosin and RuBisCO may also suggest that vanadate-dependent photomodification is selective for serine. However, this remains to be established.

Work is currently in progress to identify the tritiated serine in RuBisCO. Further characterization of the modified enzyme may lead to the identification not only of the modified serine but perhaps to its function as well.

Acknowledgments

We thank Dr. C. Cremo for her invaluable advice and assistance. We also thank Mark Reavis of the Bioanalytical Center, WSU, for performing the amino acid analysis.

Registry No. RuBisCO, 9027-23-0; RuBP, 24218-00-6; FBP, 488-69-7; V_i, 14333-18-7; Ser, 56-45-1; bicarbonate, 71-52-3; phosphate, 14265-44-2.

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