Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 865-873

EVIDENCE FOR THE INVOLVEMENT OF SUPEROXIDE ANIONS IN THE OXYGENASE REACTION OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

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Received September 7,1978

Summary The mechanism of oxygenase reaction catalysed by ribulose-1,5-diphosphate carboxylase was investigated using superoxide dismutase from bovine erythrocytes. Inclusion of superoxide dismutase in the assay mixture resulted in strong inhibition of oxygenase reaction. Ribulose-1,5-diphosphate was found to compete for superoxide anions with dismutase and nitro-blue tetrazolium which also inhibited the oxygenase reaction. These observations indicate the possible involvement of superoxide anions in the oxygenase reaction.

Ribulose-1,5-diphosphate carboxylase is a bifunctional enzyme. It catalyses the carboxylation reaction in the reductive pentose phosphate cycle and the oxidation of ribulose-1,5-diphosphate (RuDP) in photorespiration yielding phosphoglycolate and 3-phosphoglycerate (1). It is believed that the enzyme has inherent oxygenase activity and can thus account for photo-respiration. Branden (2) has recently claimed that RuDP carboxylase and oxygenase from green plants are two different enzymes based on the separation achieved using gel filtration at pH 8.3. It has been shown in case of several mono and dioxygenases (3-7) that the reactive oxygen species involved in the reaction is superoxide anion (O_2^-). Although in the case of RuDP oxygenase Wildner (8) had proposed that superoxide anions may be involved in the oxygenase reaction, so far there has been no direct evidence to indicate its involvement in the oxygenase reaction. The mechanism of oxygenase reaction is still uncertain. This communication presents evidence to demonstrate the participation of superoxide anions in the oxygenase reaction of RuDP carboxylase enzyme. Taking advantage of this observation, a simple enzymatic staining method for carboxylase/oxygenase enzyme on polyacrylamide gel is also described.

0006-291X/78/0844-0865$01.00/0

Materials and Methods RuDP carboxylase was isolated from spinach leaves. The enzyme was purified according to the method of Paulsen and Lane (9). The purity was adjudged by polyacrylamide gel electrophoresis.

RuDP oxygenase activity was determined as described previously (10). The enzyme was fully activated by addition of 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ at pH 8.5 for about 10 minutes at room temperature. The protein fraction used throughout these experiments had a specific activity of 80 nmoles of O_2 consumed per mg protein per minute.

Superoxide dismutase was purified from bovine erythrocytes according to the method of McCord and Fridovich (11). The assay of enzyme activity depended on the ability of the dismutase to inhibit the reduction of nitroblue tetrazolium (NBT) by O_2^- generated by photochemically reduced flavins. A unit of enzyme activity is defined as that amount of enzyme which causes a 50% inhibition of cytochrome c reduction.

NBT and cytochrome c reduction was assayed spectrophotometrically using Hitachi Perkin-Elmer double beam recording spectrophotometer. NBT reduction was monitored at 560 nm and cytochrome c at 550 nm. The reaction mixture in a total volume of 3 ml contained 50 mM Tricine-NaOH (pH 8.5), 10 mM $MgCl_2$, 0.3 mM NBT or 0.07 mM cytochrome c and the appropriate amounts of the enzyme.

RuDP carboxylase/oxygenase was stained on polyacrylamide gel using NBT reduction method. Electrophoresis was performed according to the method of Davis (12). RuDP carboxylase/oxygenase was localised by soaking the gel in 2.4×10^{-3} M NBT and 50 mM Tricine-NaOH (pH 8.5). After about 15-30 minutes purple to blue coloured band due to the formation of insoluble formazan is visible.

Results

Inhibition of oxygenase reaction by NBT and dismutase Sensitivity of RuDP oxygenase reaction to superoxide dismutase and NBT was measured as a function of their concentrations. As illustrated in Figs. 1 A and B there was a progressive increase in the inhibition of oxygenase reaction with the increasing concentrations of dismutase or NBT. About 80% inhibition was recorded at 0.17 mM NBT or at 80 units of dismutase addition to the reaction mixture. Cytochrome c addition to the reaction mixture also inhibited the enzyme activity. These data suggest that O_2^- is involved in the oxygenase reaction. The validity of the dismutase inhibition of oxygenase reaction implicating the involvement of superoxide anions in the oxygenase reaction was tested by using dismutase that was inactivated either by boiling the enzyme (10 min. at 100°C) or by removal of Cu^{++} using 1×10^{-3}M EDTA at pH 3.8 (13). It was made sure that such a treated enzyme was completely devoid of

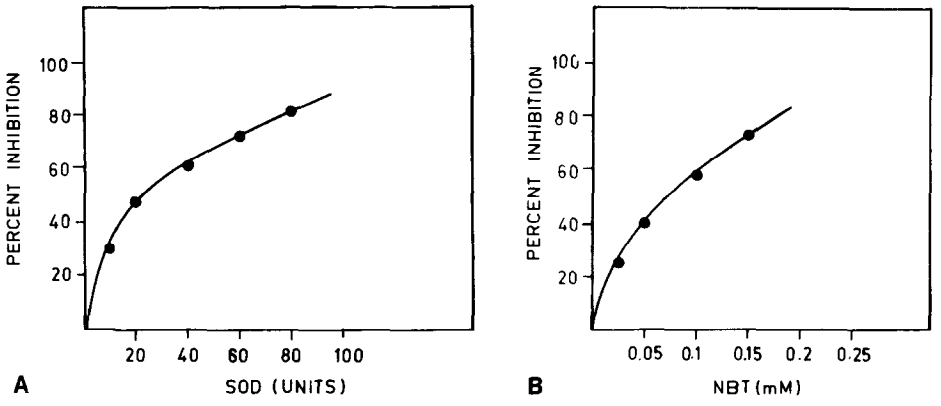


Fig. 1(A) Effect of varying concentrations of superoxide dismutase on RuDP oxygenase reaction. The reaction mixture in 1.5 ml contained 50 mM Tricine-NaOH (pH 8.5), 10 mM $MgCl_2$ and 250 µg of activated enzyme protein. The reaction was started by addition of RuDP to a final concentration of 0.6 mM. (B) Effect of varying concentrations of NBT on RuDP oxygenase reaction. The indicated concentrations of NBT were included in the assay mixture.

its catalytic function by using standard assay methods. The results presented in Table I show that the enzyme which has been made completely devoid of its capacity to scavange O_2^- did not inhibit the oxygenase activity.

Role of sulfhydryl groups in O_2^- production Sulfhydryl groups are implicated in O_2^- production. To test this, the enzyme was preincubated with sulfhydryl blocking agents like iodoacetamide (IAA) or N-ethyl maleimide (NEM) at 5 and 2 mM concentrations respectively. It was observed (Table II) that these inhibitors while affecting the oxygenase reaction also inhibited cytochrome c and NBT reduction catalysed by RuDP oxygenase enzyme considerably, indicating the role of sulfhydryl groups in NBT and cytochrome c reduction. A recent report from this laboratory (10) has shown that the oxygenase activity of the enzyme can be completely inhibited by hydroxylamine without affecting carboxylase activity. We wondered if hydroxylamine interfered with the activation of oxygen molecule. The effect of hydroxylamine on O_2^- production was therefore, checked. It was observed that hydroxylamine does not inhibit cytochrome c or NBT reduction catalysed by the enzyme (data not

Table I

Inhibition by dismutase of oxygenase reaction of RuDP carboxylase

Reaction mixture	Activity nmoles O_2 consumed/min mg protein	% Inhibition
Complete	80	Nil
Plus dismutase (60 units)	20	75
Plus boiled dismutase (100 units equivalent protein)	80	Nil
Apoenzyme* (EDTA treated at pH 3.8)	80	Nil

RuDP oxygenase reaction mixture is given in legend to Fig. 1.
* Dismutase enzyme was treated with 1 mM EDTA at pH 3.8 and dialysed. 100 units equivalent apoenzyme was added to the reaction mixture.

presented) confirming our earlier proposal that hydroxylamine inhibition may not be due to its effect on SH groups of the enzyme.

Effect of RuDP on O_2^- production RuDP strongly inhibited the enzymatic reduction of cytochrome c (Table III). This possibly means that RuDP competes for O_2^- produced in the reaction with cytochrome c. This will be supported by the dismutase data to be presented later. Reduction of cytochrome c and NBT was completely inhibited by the addition of superoxide dismutase confirming the involvement of O_2^- in the reaction (Table III).

Reversal of dismutase inhibition of oxygenase reaction by RuDP As mentioned earlier RuDP seemed to compete with cytochrome c for O_2^- produced in the reaction. This was further confirmed with the help of dismutase inhibition of RuDP oxygenase reaction. It can

Table II

Effect of preincubation of RuDP oxygenase with sulfhydryl group inhibitors on cytochrome c reduction

	Additions to the preincubation mixture[a]	NEM/IAA in assay mixture (mM)[b]	ΔA_{550}/min.	% Inhibition
1)	None	0.1	0.12	Nil
2)	None	2	0.04	66
3)	2 mM NEM	0.1	0.06	50
4)	5 mM IAA	0.25	0.05	58

a) The preincubation mixture consisted of 50 mM Tricine-NaOH (pH 8.5) and 2.5 mg of enzyme protein in a total volume of 1 ml. The variable additions were 2 mM NEM or 5 mM iodoacetamide (IAA). After 90 minutes of incubation 0.2 ml of aliquot was removed and assayed for cytochrome c reduction.
b) Levels of NEM/IAA indicated in the reaction mixture were added to the cytochrome c reduction assay before the reaction was initiated by addition of cytochrome c. In reactions 3 and 4 the concentration of NEM or IAA is due to carry over from pre-incubation stage.

Table III

Effect of RuDP and dismutase on cytochrome c reduction by RuDP oxygenase

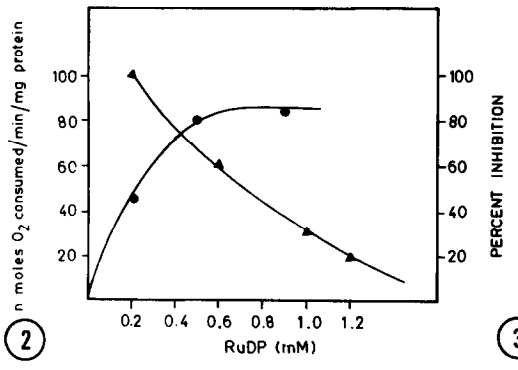
Reaction mixture	ΔA550/min	% Inhibition
Complete	0.185	Nil
Boiled enzyme	0.03	-
Plus superoxide dismutase (60 units)	0.03	85
Plus RuDP (0.6 mM)	0.07	62

Cytochrome c reduction was measured spectrophotometrically as described in Materials and Methods.

be seen from the data presented in Fig. 2 that dismutase inhibition was reversed by increasing the RuDP concentration when the concentration of dismutase used in the experiment caused 50% inhibition of oxygenase reaction under standard assay conditions. Lowering the RuDP concentration in the assay increased the ability of dismutase to inhibit the oxygenase reaction and vice-versa.

Staining of RuDP carboxylase/oxygenase on polyacrylamide gel

To stain the enzyme on polyacrylamide gel, the advantage is taken of the fact that the enzyme reduces NBT in absence of the substrate. The photograph of such a stained gel is presented in Fig. 3 along with a gel which has been stained for protein. The purified enzyme preparation was run on gel columns A and B and the ammonium sulphate fraction between 30-45% saturation was run in gel column C. In gel column A bands were localized using protein stain coomassie brilliant blue. Gel columns B and C were stained enzymatically using NBT reduction method. It was observed that band due to NBT reduction corresponded to the protein band of RuDP carboxylase/ oxygenase enzyme.



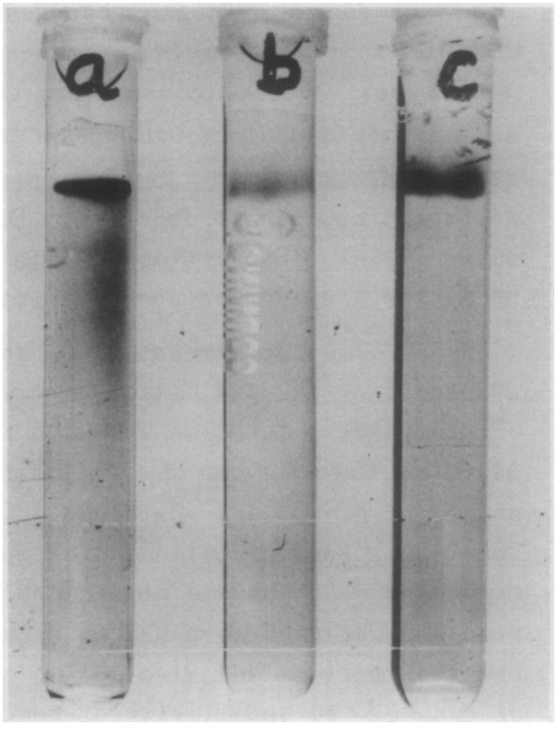


Fig. 2. Effect of RuDP on inhibition of oxygenase activity by superoxide dismutase. Reaction mixture was as described in Fig. 1.
● —— ● Control reactions showing nmoles of O_2 consumed in the absence of dismutase at varying concentrations of RuDP.
▲——▲ Percent inhibition of oxygenase reaction in the presence of 20 units of dismutase at varying concentrations of RuDP.

Fig. 3 Polyacrylamide gel electrophoresis pattern of RuDP carboxylase/oxygenase enzyme. (A) Purified enzyme preparation stained for proteins. (B) Purified preparation stained for the enzyme using NBT reduction method. (C) Ammonium sulphate fraction (30-45%) stained for oxygenase by NBT reduction method.

Discussion The reaction catalysed by RuDP oxygenase was found to be sensitive to the addition of NBT and dismutase. Lorimer et al (14) were unable to demonstrate any inhibitory effect of dismutase on oxygenase activity. This was based on the observation that no change in the extent of inhibition by dismutase was noticed after the dismutase was supposedly inactivated by heat. The reasons for the failure to observe inhibition by dismutase by them could be due to insufficient boiling time to inactivate

dismutase enzyme. It was observed by us and also by others (3) that 10 minute boiling time is necessary to completely inactivate the enzyme. Also no checks were made, atleast none reported, to see whether dismutase has been completely inactivated when boiled for 2 min. at 100°C. In our experiments we had used two different methods to inactivate the dismutase enzyme and in each case complete inactivation of the enzyme was confirmed before using the preparation for inhibition studies.

The involvement of O_2^- in the oxygenase reaction is evidenced by the inhibition of oxygenase reaction on the addition of dismutase. Catalase was also used in combination with dismutase to rule out the inhibition due to H_2O_2 produced by the action of dismutase. Catalase as such did not inhibit oxygenase reaction. Further evidence for generation of superoxide in the oxygenase reaction was obtained by a) Inhibition of oxygenase reaction observed when NBT or cytochrome c were added to the reaction mixture and also due to the observed enzymatic reduction of cyt. c. b) Sensitivity of reduction of this dye to the addition of dismutase.

Wildner (8) had proposed a scheme for oxygenase reaction in which a cysteine residue of the active centre is 'activated' to thiyl radical and oxygen is reduced to superoxide anion. O_2^- was thought to be the active oxygen species which react with C-2 radical on the RuDP molecule in the oxygenase reaction. The experimental evidence for this scheme comes for the first time from our observations that RuDP competes for the O_2^- with dismutase, a potent scavenger of this radical, NBT and cytochrome c. The inhibition of O_2^- production by sulfhydryl blocking agents also supports the hypothesis that SH groups may be involved in the activation process.

The data presented in this communication suggest that in the case of RuDP oxygenase, superoxide radical anions are involved in oxygenation reaction. The mechanism of O_2^- production is not clear but it is likely that Cu^{++} has a role in the process.

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