The Activation of Ribulose-1,5-bisphosphate Carboxylase-Oxygenase from Spinach by Oxygen

Changes of the Enzyme Conformation during Air-Argon Transitions

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The effect of oxygen on ribulose-1,5-bisphosphate carboxylase-oxygenase from spinach was investigated. Both activities were deactivated by removal of oxygen and reversibly reactivated by oxygenation of the enzyme solution. The change in enzyme activities was accompanied by conformational changes as studied by the use of intrinsic and extrinsic fluorescent probes.

The analysis of cysteine sulfhydryl groups accessible to 5,5′-dithiobis-(2-nitrobenzoic acid) revealed that the number of these groups changed with the oxygen concentration. The kinetic of the exposure of eight cysteine residues was similar to the loss of enzyme activities. The modification of these groups with 5,5′-dithiobis-(2-nitrobenzoic acid) caused almost complete loss of both the activities.

The enzyme isolated from a photolithotrophic organism, *Chromatium vinosum*, was not affected by oxygen removal. During air – argon transitions, neither the enzyme conformation nor the number of accessible sulfhydryl groups changed.

Introduction

Ribulose-1,5-bisphosphate carboxylase can be preactivated in the presence of the substrate CO_2 and Mg^{2+} (*cf.* a recent review by Jensen and Bahr [1]). This preactivation reaction presumably modifies the ε-amino group of one of the lysine residues to a carbamate [2, 3] to which Mg^{2+} ions are quickly bound [2]. In recent studies by Miziorko [4] the binding of CO_2 to the enzyme was further characterized. The enzyme bound CO_2 was isolated in presence of carboxyribitol bisphosphate yielding a stoichiometry of one activator site to one catalytic site [4]. Although the mechanism for the carboxylation reaction has been partially elucidated [5–7], the information on the function of the activating site in the enzyme catalysis is still missing.

RuBP carboxylase as a bifunctional enzyme has oxygenase activity; RuBP is oxidized to one molecule of phosphoglycolate and one molecule of 3-phosphoglycerate. The incubation of the enzyme with CO_2, the substrate for carboxylase, and Mg^{2+} preactivates the oxygenase in the same manner as the carboxylase [8].

In a recent preliminary publication we reported that RuBP carboxylase-oxygenase also requires activation by O_2 in addition to CO_2 and Mg^{2+} [9]. The removal of oxygen caused a reversible loss of both enzyme activities. The enzyme can gain full activity only in the presence of CO_2, O_2 and Mg^{2+}.

This study reports the change of the enzyme conformation due to the transitions from aerobic to anaerobic conditions. Furthermore, the analysis of the sulfhydryl groups revealed that under anaerobic conditions and in the presence of Mg^{2+} the enzyme has more cysteine residues exposed than in the presence of oxygen. The observation that both enzyme activities, carboxylase and oxygenase, are affected similarly, suggests that a common step of both enzyme reactions (*i. e.* binding or enolization of RuBP) is dependent on the oxygen activated enzyme molecule.

Abbreviations: RuBP, ribulose-1,5-bisphosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); ANS, 1-anilino naphthalene-8-sulfonate; PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecylsulfate.
Enzymes: RuBP carboxylase – oxygenase: 3-phospho-D-glycerate carboxy-lyase (dimerizing) (EC 4.1.1.39).

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Methods and Materials

Isolation of RuBP carboxylase – oxygenase from spinach

The enzyme was isolated by grinding spinach leaves in 50 mM Tris-H_2SO_4 buffer, pH 8.0, contain-

ing 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 2 mM EDTA and 2 mM 2-mercaptoethanol. The purification process included the following steps: ammonium sulfate precipitation (30 to 55% saturation); Sephadex G-200 gel filtration and ultracentrifugation on a sucrose step gradient; details of the procedure have been described elsewhere [10]. The enzyme fractions with the highest specific activity (carboxylase: 1.2 U/mg protein, oxygenase: 0.12 U/mg protein) were collected and used throughout the experiments. This fraction showed a single band in 5% acrylamide gels after disc electrophoresis [11].

The enzyme was kept in the activated state (in presence of 10 mM $NaHCO_3$ and 10 mM $MgCl_2$) during the isolation process and stored in 30% sucrose at −15 °C. Under these conditions the enzyme activity was stable for at least four months.

Isolation of RuBP carboxylase – oxygenase from Chromatium vinosum

Chromatium vinosum strain D (DSM 180) was grown photolithoautotrophically in a medium described by Pfennig and Lippert [12] at approximately 5000 lx and 25 °C. The cells, grown in three 10 l carboys, were harvested at the late growth phase by centrifugation and washed once with 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$; the yield was 95 g of wet packed cell material. After suspension in 300 ml of 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $NaHCO_3$, 10 mM $MgCl_2$ and 2 mM 2-mercaptoethanol, the cells were sonicated for 3 min with a Branson Sonifier B 15 (50% duty cycle). The sonicate was centrifuged at $35000 \times g$ for 30 min and the enzyme in the supernatant was precipitated by ammonium sulfate. The precipitate which was derived at a saturation range of 35 to 60% was dialysed against the Tris buffer with 2-mercaptoethanol overnight. The dialysate was centrifuged for 2 h at $210000 \times g$ to clarify the protein solution from membrane vesicles. The supernatant was applied to Sephadex G-200 column (3.5 × 70 cm). The enzyme fractions with the highest specific activities were further purified by sucrose density gradient centrifugation as described earlier [10]. The fractions were collected and analysed for protein content, enzyme activities and homogeneity. The electrophoresis was carried out according to Williams & Reisfeld [13] with the Tris-diethylbarbiturate buffer system, pH 7.0. The gels showed one major band with a minor band of higher molecular weight. The specific activity of the RuBP carboxylase was equivalent to 1.4 U/mg protein, and 0.14 U/mg protein for the RuBP oxygenase.

RuBP carboxylase and oxygenase assay

The enzyme was dialysed against 50 mM Tris-H_2SO_4 buffer, pH 8.0, with additions as indicated in the legends to the tables and figures. The dialysed enzyme (about 15 mg protein/ml) was gassed with argon in an oxygraph cell (Rank Brothers, Cambridge, England) at 25 °C with a gas flow of 0.5 to 1 l/h. The oxygen content of the cell was monitored continuously.

Prior to the enzyme assays, the enzyme solution was adjusted to 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ to achieve full activation. The assay mixture for the carboxylase assay contained in 0.2 ml: 50 mM Tricine-NaOH buffer, pH 8.0; 10 mM $MgCl_2$; 1 mM RuBP; 25 mM $NaH^{14}CO_3$ (1.9×10^{10} Bq/mol) and enzyme protein equivalent to 10 µg of protein. The reaction was stopped after 2 min by the addition of 0.05 ml glacial acetic acid, and aliquots were removed for scintillation counting.

The oxygenase assay mixture contained in 1.5 ml: 50 mM Tris-H_2SO_4 buffer, pH 8.5, 10 mM $MgCl_2$, 0.7 mM RuBP, 0.26 mM O_2 and enzyme equivalent to 0.1 mg of protein and 0.35 mM bicarbonate which was carried over with the enzyme solution. The reaction was started by the addition of the enzyme solution, and the oxygen uptake was measured polarographically (oxygraph by Rank Brothers, Cambridge, England).

In the experiments, where CO_2-free conditions were important special precautions were taken to remove CO_2 from all solutions as described earlier [14].

Measurements of intrinsic protein fluorescence

The protein fluorescence emission was recorded with an Aminco SPF-500 spectrofluorometer. The excitation and emission spectra indicated maxima at 284 nm (excitation) and 336 nm (emission). At these wavelengths all the measurements were carried out with enzyme samples of 0.3 mg of protein/ml.

Measurement of ANS-fluorescence

The enzyme samples (0.3 mg of protein/ml) were titrated with small portions of 20 nmol ANS and the

fluorescence emission intensity was measured at 495 nm at 25 °C; the fluorescence excitation occured at 365 nm following a procedure described earlier [10].

Circular dichroism measurements

The CD-spectra were recorded in the wavelength region of 200 – 305 nm with a dichrographe III (CNRS-Roussel-Jouan) linked to a digital PDP-8 minicomputer. The enzyme samples (1.06 mg protein/ml of 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$) were gassed with either air or argon for 3 hours at 25 °C. The spectra were recorded at room temperature with cuvettes of 0.2 cm path length.

Sulfhydryl group analysis

a) DTNB: The titration method followed was basically that of Takabe and Akazawa [15] with the Ellman's reagent. Complete unfolding of the protein chains was achieved by using 5% SDS as detergent for 30 min at 25 °C. The number of the DTNB modified sulfhydryl groups per mol enzyme was calculated with a molar extinction coefficient of 12930 using cysteine as a standard.

b) Aldrithiol-2 (2,2′-dithiodipyridine): The experimental procedure was based on the method of Grassetti and Murray [16] with minor modifications. The reagent was dissolved in 100 mM phosphate buffer, pH 7.0. The enzyme solution contained in 1,5 ml: 50 mM Tris-H_2SO_4 buffer, pH 8.0, 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ and protein in the range of 1.5 to 4 nmol. A molar extinction coefficient of 7300 using cysteine as a standard was employed.

c) PCMB: The titration of sulfhydryl groups with *p*-chloromercuribenzoate was carried out according to the method of Boyer [17], as modified by Sugiyama *et al.* [18]. A two hundredfold excess of PCMB was allowed to react with 3 nmol of enzyme protein for 60 min at 25 °C and the increase in absorbance at 250 nm was measured. The value of 550000 daltons for the molecular weight of the spinach enzyme was used for all the calculations.

Results

Conformational studies

The removal of oxygen from a solution containing purified spinach fraction I protein not only caused a severe decrease of RuBP carboxylase and oxygenase activity [9] but also a significant change of the conformation of the enzyme molecules. The changes as affected by the incubation conditions (RuBP, CO_2, O_2, Mg^{2+} and Ar) were studied (i) by the measurement of the intrinsic protein fluorescence, (ii) by the use of the fluorescent probe ANS, and (iii) by circular dichroism.

The purified spinach enzyme (15 mg protein/ml 50 mM 50 mM Tris-H_2 SO_4 buffer, pH 8.0) was preincubated either with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ or with 2 mM RuBP and 10 mM $MgCl_2$ for 3 hours and the emission spectra of both samples, diluted to 0.3 mg protein/ml, were recorded (Fig. 1). Both samples were gassed simultaneously for 3 hours with argon and after the complete removal of oxygen was obtained, they were used for the fluorescence analysis. All the excitation and emission curves had the same maximum at 284 nm and 336 nm respectively. The sample pretreated with CO_2 and Mg^{2+} and exposed to argon had a considerably lower fluorescence yield as compared to the sample exposed to air. On the contrary the samples pretreated with RuBP and Mg^{2+} always had the same fluorescence emission independent of aerobic or anaerobic conditions. In our opinion, the observed changes in the fluorescence intensities reflect conformational changes of the enzyme molecules, as discussed later.

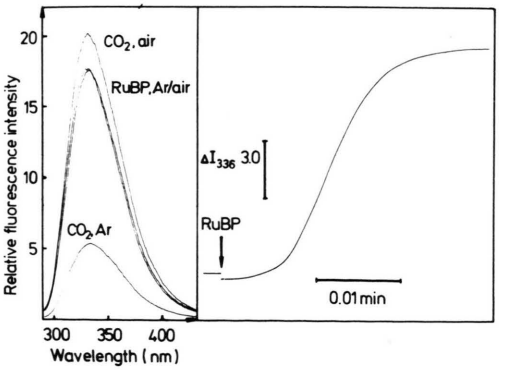

Fig. 1. Emission spectra and kinetic of change in protein fluorescence of RuBP carboxylase-oxygenase. Dialysed spinach enzyme was gassed with air or argon for 3 hours at 25 °C either in presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ or 10 mM $MgCl_2$ and 2 mM RuBP. Both samples were diluted to 0.3 mg protein/ml with the appropriate buffers and the fluorescence emission spectra were recorded. The excitation occured at 284 nm. The kinetic of fluorescence rise was measured with an anaerobic sample in buffer containing 10 mM $MgCl_2$ after the addition of 1 mM RuBP.

Table I. Intrinsic protein fluorescence of RuBP carboxylase-oxygenase. The dialysed spinach enzyme (50 mM Tris-H_2SO_4 buffer, pH 8.0) was incubated for 3 h at 25 °C with substrates and $MgCl_2$ as indicated; parts of the samples were gassed with argon. The intrinsic protein fluorescence was measured at 336 nm; the excitation occured at 284 nm.

Experimental conditions	Relative fluorescence intensity	
	air	argon
Dialysed enzyme fraction	19.1	21.3
plus 10 mM $NaHCO_3$	19.3	19.5
plus 10 mM $MgCl_2$	19.9	4.9
plus 10 mM $MgCl_2$ + 10 mM $NaHCO_3$	20.1	5.3
plus 1 mM RuBP	18.4	17.8
plus 1 mM RuBP + 10 mM $MgCl_2$	17.6	17.6
plus 1 mM RuBP + 10 mM $NaHCO_3$	17.9	18.0

These studies were extended to investigate the influence of the substrates RuBP, CO_2 and O_2, as well as Mg^{2+}, either singularly or in combination, on the fluorescence emission of the enzyme molecules (Table I). The enzyme solutions were incubated with the additions as listed in Table I for 3 hours at 25 °C in air, and part of the incubation mixtures were gassed with argon. The results indicate that only the anaerobic samples with Mg^{2+} alone or with Mg^{2+} and CO_2 show a definite change compared with the air-exposed samples. As already mentioned above, the samples incubated with Mg^{2+} and RuBP did not show this drastic change (see Fig. 1). The sequence of the addition of either RuBP or $MgCl_2$ to the enzyme solution had no influence on the fluorescence emission. The addition of 1 mM RuBP to an anaerobic enzyme solution preincubated with 10 mM $MgCl_2$ caused the rise of the fluorescence intensity. The kinetic of the change is presented in Fig. 1; half of the enzyme molecules became converted in 0.7 s after the addition of RuBP and complete conversion was achieved after 1.4 s.

The smaller differences in the fluorescence intensity of the samples with different additions (Table I) have not been considered significant, although they probably indicate minor conformational changes.

The kinetic of the conformation changes of the enzyme molecule due to air – argon – oxygen transitions was studied (Fig. 2). The enzyme solution, preactivated with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$, was gassed with argon and samples were continuously removed to monitor the intrinsic pro-

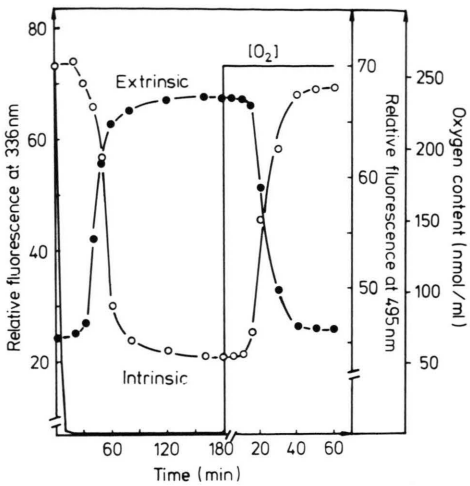

Fig. 2. Changes in intrinsic and extrinsic fluorescence of RuBP carboxylase-oxygenase due to air-argon-oxygen transitions. Fully activated spinach enzyme was gassed with argon for 3 hours at 25 °C, then the gas flow was switched to oxygen. At the beginning of the experiment the oxygen content of the oxygraph cell was 258 nmol/ml and it was monitored continuously. Aliquots were removed and the fluorescence emission was measured at 336 nm (●–●). The extrinsic fluorescence was determined by adding the enzyme samples to 0.09 mM ANS and the fluorescence emission was recorded at 495 nm (○–○).

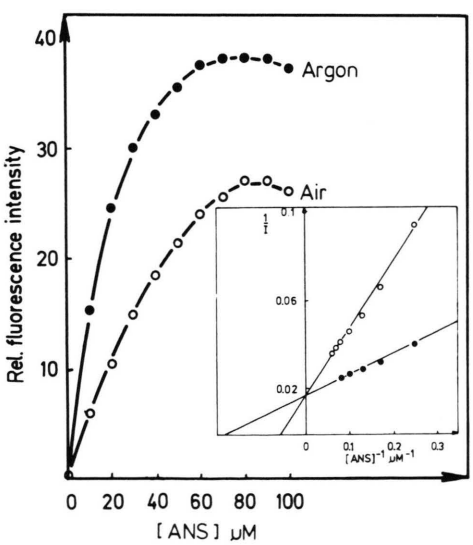

Fig. 3. Changes of extrinsic (ANS) fluorescence of aerobic and anaerobic RuBP carboxylase-oxygenase. Aerobic (○–○) and anaerobic (●–●) spinach enzyme samples (0.6 mg protein in 2 ml 50 mM Tris-H_2SO_4, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$) were titrated with small portions of 20 nmol ANS at 25 °C and the fluorescence emission was measured at 495 nm. The insert shows the double reciprocal plot 1/fluorescence intensity versus 1/(ANS).

tein fluorescence. This curve shows a definite lag phase between the oxygen removal and the onset of the fluorescence change. A similar effect could be observed during the transition from argon to oxygen; 15 min after the oxygenation of the solution the fluorescence emission increased again and nearly reached the original level after 60 min.

This major protein conformation change was also observed by the use of the extrinsic fluorescence probe ANS. The enzyme solution (0.6 mg protein in 2 ml of 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$) was kept either aerobic or anaerobic with argon. Both samples were titrated with ANS and the fluorescence emission was registered (Fig. 3). The fluorescence emission of the anaerobic sample was considerably higher than that of the aerobic sample. The fluorescence emission maximum in all the cases, however, remained unchanged at 495 nm. The insert of Fig. 3 shows the double reciprocal plot 1/fluorescence intensity versus 1/(ANS). The maximal fluorescence intensity was equal for both samples; the difference between the two of them was confined to the change in the affinity of ANS to the enzyme molecules. These results provided further evidence to support the assumption that during air – argon transitions a major conformation change of the preactivated enzyme molecules occurs. The kinetic of this change of the fluorescence emission from the low fluorescent state (aerobic condition) to the high fluorescent state (anaerobic condition) was measured and the results are included in Fig. 2. A retardation of the onset of conformation change was observed with the fluorescence probe ANS which was similar to that of the intrinsic protein fluorescence described above.

Circular dichroism spectroscopy was also employed to study the conformational changes. This method as a probe in studying conformational changes of proteins is proving to be a valuable although not unambigous technique [19]. The observation of a change in optical rotation could indicate conformational changes; however, the converse does not support the lack of such changes, especially if the protein chains are folded to a minor extent in α helices as seen at low molecular ellipticity values in the region of 210 to 225 nm [20].

The spectra in the range of 200 – 305 nm of the air and argon treated RuBP carboxylase – oxygenase samples show only minor differences (Fig. 4).

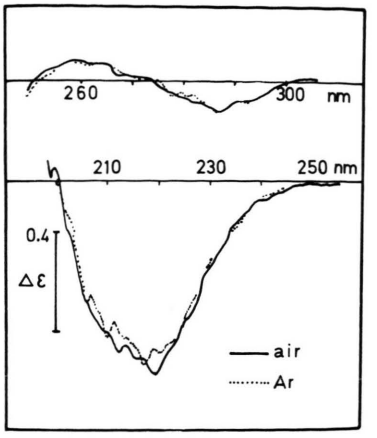

Fig. 4. Circular dichroism of spinach RuBP carboxylase-oxygenase. Purified enzyme (1 mg protein in 1 ml 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $MgCl_2$ and 10 mM $NaHCO_3$) was kept either aerobic (——) or gassed for 3 hours with argon (——). The aerobic sample had the highest $\Delta\varepsilon$ of −0.78 at 219 nm with smaller ellipticity bands of $\Delta\varepsilon$ −0.695 at 212.7 nm and $\Delta\varepsilon$ of −0.012 at 287 nm. The argon treated sample had $\Delta\varepsilon$ at 217.5 nm of −0.725, at 210 nm of −0.629 and at 286 nm −0.012.

The spectra of the aerobic sample had a ellipticity band at 219 nm = -2.57×10^3 deg × cm²/dmol; the argon treated sample had a ellipticity band at 217.5 nm = -2.39×10^3 deg · cm²/dmol. Recent studies by Grebanier *et al.* [21] in the region of 250 – 320 nm could also demonstrate only minor changes in the spectra of non- and preactivated RuBP carboxylase.

Sulfhydryl group analysis

The removal of oxygen from RuBP carboxylase – oxygenase caused, in addition to the loss of enzyme activities [9], a significant change of the enzyme conformation. Therefore, it was of interest to study whether this change also affected the accessibility of the sulfhydryl groups of the cysteine residues of sulfhydryl reacting reagents. Spinach RuBP carboxylase has a large number of 96 cysteine residues; 9 of them were estimated for the large subunit and 3 of them for the small subunit [15]. The accessibility of the cysteine residues was dependent on the chemical nature of the analysing reagent [15]. Two compounds, DTNB and 2,2′-dithiodipyridine, which react only partially with the enzyme protein and one compound, PCMB, which reacts rather quantitatively, were selected.

Table II. Sulfhydryl group analysis with DTNB. Dialysed spinach enzyme (15 mg/ml 50 mM Tris-H_2SO_4 buffer, pH 8.0) was incubated either aerobically or anaerobically for 3 h at 25 °C. Part of the samples contained 10 mM $MgCl_2$ or 10 mM $MgCl_2$ plus 10 mM $NaHCO_3$. Aliquots were removed and sulfhydryl groups were determined by the method of Takabe and Akazawa [15] with DTNB. The enzyme reacted for 30 min with a three hundred-fold excess of DTNB. Complete denaturation of the enzyme molecules occured in presence of 5% SDS.

Experimental conditions	SH groups/mol enzyme			
	air		argon	
	native	denaturated	native	denaturated
Dialysed enzyme fraction	11.8	94.2	11.4	94.8
plus 10 mM $MgCl_2$	11.2	94.5	19.5	96.3
plus 10 mM $MgCl_2$ + 10 mM $NaHCO_3$	11.3	95.7	19.4	93.9

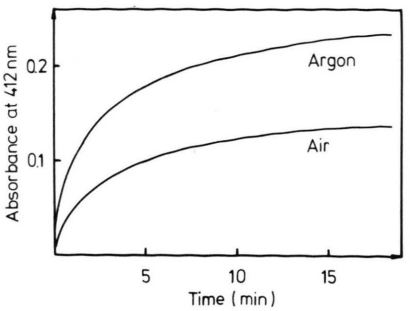

Fig. 5. Kinetic of the spinach enzyme reaction with DTNB. Fully activated enzyme samples were gassed with argon or air for 3 hours at 25 °C. The reactions were carried out according to the method of Takabe and Akazawa [15]. The increase of the absorption at 412 nm was monitored.

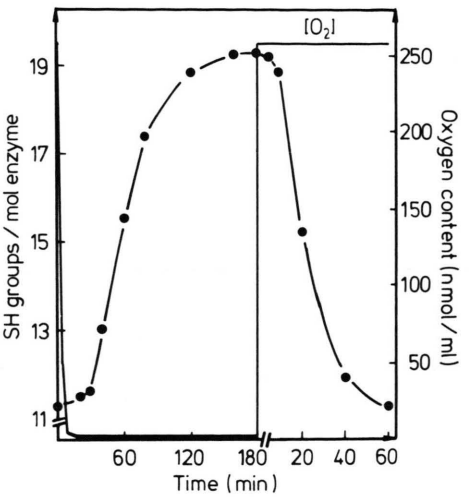

Fig. 6. Kinetic of the exposure of cysteine residues during air-argon transition. Fully activated spinach enzyme was gassed with argon for 3 hours at 25 °C followed by reoxygenation of the enzyme solution. At definite intervals aliquots were removed and the number of sulfhydryl groups (●–●) was determined with DTNB by the method of Takabe and Akazawa [15]. The oxygen content during the incubation was continuously monitored (——).

An enzyme solution, preactivated with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ was used for the analysis of the cysteine residues with DTNB according to the method of Takabe and Akazawa [15]. Under aerobic conditions and with a reaction time of 30 min, only about 11 – 12 out of a total of 96 cysteine residues could react with this reagent (Table II). The transition from aerobic to anaerobic conditions triggered a change of the enzyme conformation, which was reflected by an increase in the number of accessible cysteine residues. These groups could react with DTNB with the same reaction time under similar conditions. Unfolding the protein chains by the use of the detergent SDS made possible the measurement of the total number of cysteine residues, which remained unchanged as about 96 groups.

The kinetics of the reaction between DTNB and the aerobic and anaerobic protein sample are presented in Fig. 5. Both reactions were almost complete after 15 min; afterwards, a slower reaction was observed.

The method of sulfhydryl group titration with DTNB was employed to study the kinetics of their exposure during the air – argon – oxygen transitions (Fig. 6). After a lag phase of 20 min the appearance of the additional accessible eight sulfhydryl groups was observed. An oxygenation of the enzyme solution was not paralleled by the immediate disappearance of these additional groups. The original number of 11 residues was not registered before 60 min.

Therefore, this phenomenon of delayed response to the quick gas transition was not only characteristic for the alteration of the accessibility of the cysteine residues, but also for the change of fluorescence emission and the catalytic activities of the enzyme molecules.

Since the accessibility of the sulfhydryl groups is also dependent on the chemical properties of the

sulfhydryl group reacting reagents, the analysis was repeated with two other reagents. The estimation of the cysteine residues was carried out with 2,2'-dithiodipyridine according to the method of Grassetti and Murray [16]. This method gave the same results as those obtained with the previously described procedure with DTNB: under anaerobic conditions eight additional cysteine residues were detected.

A major difference was observed with PCMB following the procedure by Sugiyama *et al.* [18]. After an incubation period of 60 min in presence of PCMB the dectable number of cysteine residues was much higher (49 cysteine/mol enzyme), compared to the rather small number of 11 cysteines detected with DTNB. Further incubation (up to 3 hours) of the enzyme with PCMB completed the modification of all the cysteine residues of the enzyme. Nevertheless, the use of PCMB in these air – argon transition experiments showed the same tendency as observed with DTNB.

The question arose whether the functions of the enzyme molecules were affected by the modification of the additional eight cysteine residues exposed by the argon treatment. Spinach enzyme (17.9 mg protein in 1 ml 50 mM Tris-H_2SO_4 buffer, pH 8.0, containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$) was incubated for 30 min at 25 °C with 5.5 mM DTNB. The reaction was stopped by passing the enzyme solution through a Sephadex G-25 column (1,5×13). The enzyme eluate was gassed with argon for 3 hours and then DTNB was added to the anaerobic solution to yield a final concentration of 5.5 mM. After 30 min the excess of DTNB was removed by gel chromatography (Sephadex G-25) and the eluate oxygenated for 60 min. At each step aliquotes were taken and assayed for enzyme activities (RuBP carboxylase and oxygenase) and for protein content; the results are summerized in Table III.

Table III. Change of enzyme activities by modification with DTNB. Preactivated spinach enzyme was treated with 5.5 mM DTNB for 30 min at 25 °C. Afterwards the modified enzyme was purified from the excess of DTNB and gassed with argon for 3 hours. A second DTNB treatment followed under anaerobic conditions; the enzyme was incubated for 30 min with 5.5 mM DTNB. After the separation of the excess of DTNB the modified enzyme was oxygenated for 60 min.

Experimental conditions		Enzyme activity	
		RuBP carboxylase U/mg protein	RuBP oxygenase U/mg protein
native enzyme	air	1.14	0.11
first DTNB treatment	air	0.96	0.08
	argon	0.06	0.005
	oxygen	0.86	0.08
second DTNB treatment	argon	0.034	0.003
	oxygen	0.08	0.008

The first treatment of the enzyme with DTNB caused a loss of 16% of the original activity for the carboxylase, and 27% for the oxygenase respectively. The anaerobic treatment diminished further both activities as expected by 95% for the carboxylase and by 95% for the oxygenase. Reoxygenation of these samples showed that the loss of the enzyme activities was only temporary and influenced by the lack of oxygen in the solution. A second incubation of the enzyme with DTNB under anaerobic conditions caused an almost complete loss of the enzyme activities: 97% for the carboxylase and 97.3% for the oxygenase. This sample has permanently lost most of its activities since the oxygenation of the enzyme solution was not correlated to an adequate increase of both activities.

Gas transition experiments with RuBP carboxylase from Chromatium vinosum

It was of special interest to study, whether the RuBP carboxylase-oxygenase activities of an anaerobic organism are also affected by gas transition experiments. The enzyme was isolated from *Chromatium* cells and purified further. Similar experiments were carried out as described above with the purified spinach enzyme. The enzyme solution was gassed either with air or with argon, and both enzyme activities were measured (Fig. 7).

The treatment of the enzyme with argon did not affect its activities, whereas the exposure to oxygen caused a severe loss. The change to argon again was related with an increase of both activities. This effect is, as expected, opposite to the observed reactions with a RuBP carboxylase of an aerobic organism.

The analysis of the sulfhydryl groups with DTNB gave the same values of 14 cysteine residues per mol enzyme for both samples (air and argon). Denaturation of the enzyme under argon and subsequent

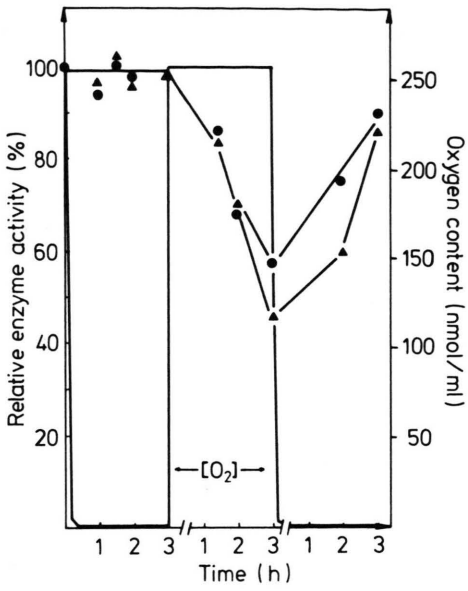

Fig. 7. Effect of gas transitions on the RuBP carboxylase-oxygenase of *Chromatium vinosum*. Fully activated enzyme (5 mg/ml in 50 mM Tris-H_2SO_4, pH 8.0, was gassed at 25 °C first with argon then with oxygen and finally again with argon 3 hours each time. Aliquots were removed at appointed times to determine, RuBP carboxylase activity (●–●) and RuBP oxygenase activity (▲–▲). During the experiment the oxygen content of the protein solution was also recorded (——).

determination of the SH-groups yielded 64 groups per mol enzyme, a value which corresponds to the data of Takabe and Akazawa [15].

The analysis of the fluorescence emission data revealed that no change had occured during the gas exchange from air to argon or vice versa.

Discussion

Preliminary studies [9] of the effect of oxygen on spinach RuBP carboxylase – oxygenase revealed that in the absence of oxygen both enzyme activities were significantly decreased. These changes were associated with alterations of the enzyme conformation as evidenced by analysis of intrinsic and extrinsic fluorescence emission (this communication).

Studies of fluorescence emission have proved useful for protein conformation analysis [22, 23]. The fluorescence spectrum of proteins due to their aromatic amino acid content, is markedly dependent on environmental quenching factors and upon possibilities for energy transfer between chromophores. The change in the fluorescence quantum yield can be correlated with alterations of the "environment", indicating changes of the conformation of the protein molecules.

Extrinsic probes such as ANS have also been used to monitor conformational changes of the proteins [22–26]. The use of this fluorescent probe was already advantageous for the studies of conformational changes of RuBP carboxylase induced by temperature variations [10, 27] and by the addition of Mg^{2+} ions or substrates [28, 29].

Our studies with both intrinsic protein fluorescence and extrinsic ANS probe showed that the transition of the enzyme molecules from one conformation to another was linked solely to the removal or addition of oxygen. Therefore, the interaction of enzyme molecules with oxygen seems to cause the observed changes. Two possibilities for explaining this effect can be considered. First, the presence of oxygen affects directly the fluorescence quantum yield of the chromophore. Second, oxygen binds to the enzyme in an unknown way, and this reaction is linked to a conformational change causing secondary effects on the chromophores.

The first explanation can be ruled out by the argument that oxygen has no prompt effect on the fluorescence emission, and secondly, that all measurements, including those on anaerobically treated samples, were carried out under aerobic conditions.

The second explanation that oxygen binds to the enzyme seems more likely. The existence of such binding sites on the enzyme for oxygen must be assured before we can propose a definite description of the activation and deactivation reaction in relation to this process. Despite the uncertainity of the existence of these binding sites we would suggest an amino acid as a possible candidate; since no other cofactor (like a heme group) known to bind oxygen is part of the enzyme.

The studies of the accessibility of sulfhydryl groups in the course of this investigation revealed that the removal of oxygen was associated with an exposure of eight cysteine residues. Previous studies by the group of Akazawa [15, 18] concerning sulfhydryl group modification of the spinach enzyme demonstrated that these groups are obligatorily involved in maintaining the enzyme structure. In the present case the exposure of the eight additional

sulfhydryl groups could also be considered as reflecting the alteration of the enzyme conformation. In addition, one could assume that the interaction of the enzyme with oxygen may be mediated by these sulfhydryl groups. The earlier work by Berger [30] demonstrating the binding of oxygen reversibly to sulfhydryl groups in an apolar environment strenghtens such an assumption. It is conceivable that oxygen is loosely bound to cysteine residues on the inside of the protein molecules. The loss of these bound oxygen molecules is responsible for the deactivation of the enzyme, as also for the observed conformational changes. Although all of the detectable oxygen in the solution was lost after 15 min, the enzyme was still fully active [9]. The subsequent loss of oxygen seems to be a rather slow process, as indicated by the kinetics of change in enzyme activity, conformation, and exposure of sulfhydryl groups.

The assumption that the sulfhydryl groups of cysteine mediate the interaction of oxygen with the enzyme molecules will have to be further investigated by the characterization of these reacting groups. The modification of the eight additional exposed sulfhydryl groups with DTNB under anaerobic conditions and the subsequent oxygenation of the enzyme solution results in a loss of both enzyme activities, even after oxygenation of the enzyme solution. The number of eight cysteine residues could indicate that each of the eight protomers of the enzyme participates in the enzyme activation mechanism.

Oxygen transition experiments with purified RuBP carboxylase from *Chromatium vinosum* revealed that this photolithoautotroph organism has no oxygen activation system. Since this organism can grow with CO_2 as the sole carbon source only under anaerobic conditions, the existence of such an oxygen activation system would be meaningless. Contrary to the enzyme of an photoautotrophic organism, oxygen has an inhibitory effect on the enzyme of *Chromatium.*

These results suggest that the enzyme from *Chromatium,* a A_8B_8 type enzyme [31], is very different from an enzyme of an aerobic organism. It would be interesting to study several RuBP carboxylases from different anaerobic organisms, including *Rhodospirillum rubrum,* an A_2 type enzyme [32] and enzymes of the A_6 type (*Rhodopseudomonas,* [33], *Chlorobium* [34]) to generalize the observed phenomenon.

To what extent the oxygen activation of RuBP carboxylase-oxygenase plays a role as a physiological control system is yet to be evaluated. In addition, it has to be further investigated whether this activation mechanism by oxygen is unique for the RuBP carboxylase-oxygenase or whether other enzymes (oxygenases) are similarly affected by oxygen.

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