Characteristic Features of the Regulatory Functions of the D-Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase from Spinach

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Regulation of Rubisco, Anionic Modulators, Specificity and Mechanism of Action of Dianionic Effectors

Catalysis and regulation of CO_2 fixation differ in a characteristic manner in their response to anionic modifiers and the polarity of the reaction medium. Monovalent inorganic anions inhibit catalysis and CO_2-activation of the D-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach, whereas the activity and binding of NADPH and effector sugar phosphates are affected only at appreciably higher concentrations. In contrast such modulators with a dianion structure stimulate CO_2 fixation by an increase of the affinity of the enzyme for the activator CO_2 and stabilization of the reactive carbamate. Structure-activity studies revealed a broad specificity of the enzyme for these regulatory effects. Essentially amino groups are involved in these processes. Certain organic solvents, as methanol or acetone, stimulate CO_2 fixation by a similar modification of the CO_2 activation centers, as induced by dianionic effectors. These results infer that such effects are due to a decrease of the polarity at the regulatory centers of the enzyme and a concomitant change of the pK of the active lysine responsible for the binding of the activator CO_2. A correlation of effector binding and activity demonstrates that already low, non-saturating concentrations of such modifiers induce high activation levels of the carboxylase and prevent the dissociation of the activated ternary complex. It is discussed that the central problem concerning the catalytical competence of D-ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the presence of active site directed dianionic effectors can be solved kinetically.

Introduction

Ribulose 1,5-bisphosphate carboxylases/oxygenases [3-phospho-D-glycerate carboxy-lyase (dimerizing) EC 4.1.1.39] are distinguished by marked regulatory properties [1−4]. These enzymes can be regulated either homotropically by the substrates CO_2 and ribulose 1,5-bisphosphate (RuP$_2$) or heterotropically by various chloroplast metabolites [4−14].

Ribulose 1,5-bisphosphate carboxylases/oxygenases are activated by preincubation with HCO_3^- and Mg^{2+}. The ternary complex carboxylase · $^ACO_2$ · Mg^{2+} is the active form of those enzymes. The existence of one separate regulatory binding site for CO_2 per catalytically active large subunit A of the Rubisco has been demonstrated by several authors [13, 15−18].

Effector sugar phosphates, as 6-phosphogluconate or fructose 1,6-bisphosphate, stimulate CO_2 fixation at relatively low concentrations and inhibit this process at higher concentrations competitively to RuP$_2$ [4, 7, 10]. Previous studies suggested that these agents bind to both the reaction centers of the carboxylase and to separate regulatory binding sites. Recently several authors reported evidence that such type of effectors elicit their stimulating effect through interaction exclusively in the region of the catalytic sites of the Rubisco [11, 12, 14]. It has been demonstrated that the mode of action of these agents is to enhance the affinity of the enzyme for the activator CO_2. In this manner the effectors support the activation process and stimulate CO_2 fixation. The aim of this publication is a) to investigate the characteristic features of the regulatory functions of the spinach enzyme and b) to clarify, how the Rubisco can remain catalytically competent in the presence of active site directed modifiers.

Methods and Materials

Methods

Enzyme preparations

Highly purified ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach was prepared essentially as described by Vater and Salnikow [10]. Sepharose 6 B was replaced by LKB ultrogel AcA 22. For quantitative binding studies with radioactively labelled effectors the enzyme was additionally purified by gradient centrifugation for 18 h at $132000 \times g$ in 10−30% (v/v) glycerol gradients using a Beckman SW 27-rotor. The specific activity of our preparations was $0.5−1.5\,\mu$mol CO$_2$ fixed per min and mg protein.

Assays for CO$_2$ fixation

1) Studies with effectors. The effector activity is defined as the excess carboxylation of RuP$_2$ in the presence of an effector in per cent over the carboxylation without effector which was taken as the reference and set to 100%.

Assay method a: Samples of the spinach carboxylase were preincubated for 10 min in an activation mixture containing 0.1 M bicine-KOH-buffer, pH = 8.0; variable effector concentrations; 0.1 mM NaH^{14}CO$_3$; 10 mM MgCl$_2$; 0.1 mM sodium ethylenediaminetetraacetate (EDTA) and 1 mM DTE. The carboxylase reaction was started with 0.5 mM RuP$_2$ and assayed for 15−60 s at limiting bicarbonate concentration (0.1 mM). The assays were terminated with 0.2 N HCl. Aliquots were dried and the acid stable product was determined by scintillation counting. CO$_2$ depleted buffers which were prepared similar to the procedure reported by Hatch and Jensen [11] were used for all experiments with effectors.

Assay method b: Samples of the enzyme were preincubated for 10 min in the same medium as under a and then diluted 100−200 fold into a reaction mixture containing 0.1 M bicine-KOH-buffer, pH = 8.0; 10 mM NaH^{14}CO$_3$; 10 mM MgCl$_2$; 0.5 mM RuP$_2$; 0.5 mM EDTA and 1 mM DTE. In contrast to method a the carboxylase activity was assayed at saturating bicarbonate concentration (10 mM).

2) Determination of the CO$_2$ activation constant K_{act} (CO$_2$). K_{act} (CO$_2$) which is defined as the CO$_2$ concentration producing half maximal activation of

the Rubisco was estimated according to the procedure published by Hatch and Jensen [11].

The enzyme was activated 10 min in activation mixtures containing variable NaHCO$_3$ concentrations and the effector concentrations indicated in Table I. The carboxylation reaction was initiated by adding 50 µl of an activation mixture to 450 µl reaction mixture specified under assay method b. Reaction time: 30 s.

Binding studies with effectors

1) Quantitative binding of [^{14}C-6]phosphogluconate and [^{14}C]fructose 1,6-bisphosphate to the spinach carboxylase was detected by the ultrafiltration technique. An Amicon micropartition system MPS-1 was used. After preincubation of the enzyme with effectors in the presence of 10 mM MgCl$_2$ the enzyme-ligand-complex was separated from the free ligand by centrifugation through YMB-filters at $1500 \times g$.

2) Fluorimetric binding studies were performed as published previously [10, 19]. 2-*p*-toluidinonaphthalene-6-sulfonate (TNS) was used as hydrophobic reporter group. Corrected difference emission spectra of the enzyme − TNS − and the enzyme − TNS − ligand complexes were recorded with a Perkin Elmer MPF 44 spectrofluorimeter in combination with a differential corrected spectra attachment DCSU-2.

Most experiments were performed at 25 °C, as otherwise indicated in the legends.

Synthesis of 6-phospho [^{14}C] gluconate

The effector was synthesized essentially as described Badger and Lorimer [12]. 6-Phosphogluconate was prepared enzymatically from D-U-[^{14}C] glucose using hexokinase and glucose 6-phosphate dehydrogenase, with a phosphoenolpyruvate-pyruvate kinase ATP regenerating system. The product was purified by ion exchange chromatography on sephadex QAE-25. Approx. 90% of the whole ^{14}C-label applied to the column appeared as a single peak which was free of nucleotides as checked by the absorption at 260 nm and the orcinol reaction. Further processing of the product obtained and determination of its specific activity were performed as indicated by Badger and Lorimer [12]. The ^{14}C content of the preparation was checked by scintillation counting. The 6-phosphogluconate concentra-

tion was determined from the absorbance change of NADPH at 340 nm in the 6-phosphogluconate dehydrogenase assay.

The [14C]-specific activity of the product corresponded within 5% with that of the starting material [14C]glucose (3.1 mCi/mmol).

Materials

RuP$_2$, NADPH, PLP and the effector sugar phosphates were obtained from Sigma (München/BRD), PGO from Serva (Heidelberg/BRD) and NMM from United States Biochemical Corp. (Cleveland/ USA). Anionic modifiers were commercial grade products of Sigma and Merck (Darmstadt/BRD). NaH14CO$_3$, [14C]fructose 1,6-bisphosphate and [14C]glucose were purchased from Amersham, Buchler (Braunschweig/BRD).

Results

I) The effects of monovalent anions on the catalytic and regulatory properties of D-ribulose 1,5-bisphosphate carboxylases/oxygenases

In Fig. 1 it is demonstrated that CO_2 fixation is inhibited by NaCl. The carboxylation of RuP$_2$ has been measured under limiting CO_2 concentrations (0.1 mM NaHCO$_3$; 10 mM Mg^{2+}) as a function of the
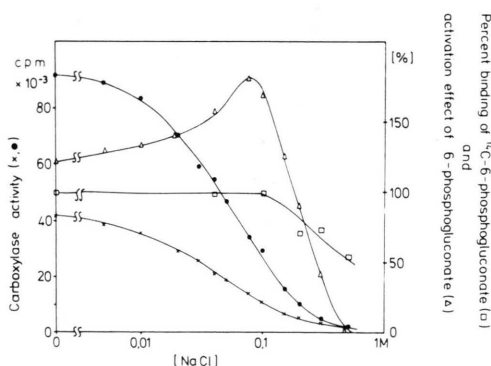

Fig. 1. Carboxylase activity in the presence of 20 μM 6-phosphogluconate (●) and without effector (×) as well as effector activity (△) and binding (□) of 6-phosphogluconate as a function of the NaCl concentration. For the measurement of CO_2 fixation assay method a was used. Reaction mixtures contained 0.1 mM NaHCO$_3$, 10 mM MgCl$_2$ and variable NaCl concentrations. The carboxylation rate in the absence of NaCl and effector was 0.21 μmol mg protein^{-1} min^{-1} (0.17 mg protein per assay). Effector binding was detected by the ultrafiltration technique.

NaCl concentration in the presence of 20 μM 6-phosphogluconate and without the effector. Under these conditions the carboxylase reaction is inhibited by the salt in the range of 10−500 mM NaCl. In contrast both the binding of [14C-6]phosphogluconate and its effector activity are more stable against NaCl inhibition than the catalytic process. They start to decline at about 0.1 M NaCl, when CO_2 fixation is already reduced to a level of approx. 25−30%. Similar results have been obtained for the effectors NADPH and fructose 1,6-bisphosphate. There is no salt effect on effector binding and activity at lower levels especially those that are comparable with the effector concentrations.

The inhibition of CO_2 fixation by various monovalent anions follows the Hofmeister series for the lyotropic properties of such agents:

$$ClO_4^- > SCN^- > CF_3COO^- > NO_3^- > Br^- > Cl^-.$$

Similar results have been obtained at salt concentrations of 20 and 200 mM. On the other hand the activity of effectors, as 6-phosphogluconate, again is not decreased under these conditions. From Table I it is apparent that various monovalent anions function as negative effectors of the carboxylase process. They increase the CO_2-activation constant K_{act} (CO_2) and, therefore, inhibit the formation of the active ternary complex enzyme · $^ACO_2 \cdot Mg^{2+} \cdot K_{act}$ (CO_2) which is defined as the CO_2 concentration for half maximal CO_2 activation of the carboxylase was estimated from double reciprocal diagrams of the CO_2 activation of the enzyme as a function of the CO_2 concentration according to the method of Hatch and Jensen [11].

Similar results as for the monovalent anions listed in Table I have been obtained also for anions of monocarboxylic acids, like acetate, propionate, butyrate, acetoacetate or ketovalerate and for sugar monophosphates, as ribose 5-phosphate or fructose 6-phosphate.

II) Studies with dianionic effectors of D-ribulose 1,5-bisphosphate carboxylases/oxygenases

1) Properties of dianionic effectors and specificity of the effector binding sites

In contrast to monovalent anions bivalent inorganic anions, like SO_4^{2-} or HPO_4^{2-} and, in particular, reagents with a dianion structure, like C2−C6 dicarboxylic acids and sugar bisphosphates with an

Table I. The maximal activity of various effectors as determined by the dilution method and CO_2-activation constants in the presence of modulators of the D-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach. For the determination of the maximal effector activity samples of the enzyme were preincubated in an activation mixture at high effector concentrations (column 2) and then diluted 200 fold into a reaction mixture, as indicated under Methods. The carboxylation rate in the absence of effector was 0.39 µmol · mg protein^{-1} · min^{-1} (0.022 mg protein in the reaction mixture). The effector concentrations (column 4) for the determination of the CO_2-activation constants refer to the maxima of effector profiles, as shown in Fig. 2.

Modulator	Effector concentration in the preincubation mixture [mM]	Effector activity [%]	Modulator concentration [mM]	K_{act} (CO_2) [µM]
–	0	0	0	71
A) Effectors:				
6-phosphogluconate	0.5	408	0.02	1.8
glucarate	50	388	2	1.6
fructose 1,6-bisphosphate	0.2	130	0.2	13
NADPH	5	420	1	2.1
sulfate	100	326	5	6
phosphate	100	363	5	9
oxalate	50	26	5	23
malonate	100	126	10	15
succinate	250	257	20	11
glutarate	250	273	20	15
2-hydroxy-glutarate	100	184	5	37
2-hydroxy-2′-methyl-glutarate	100	245	5	10
maleate	100	261	5	2.5
L-malate	200	322	5	7.5
D-malate	100	290	5	5
D,L-tartrate	20	335	2	6.3
phosphoenolpyruvate	80	269	0.5	3.7
oxalacetate	100	361	5	2.3
α-ketoglutarate	20	102	20	14.3
B) Inhibitors:				
NaCl	–	–	50	286
NaNO$_3$	–	–	50	345
NaSCN	–	–	20	400
NaClO$_4$	–	–	20	400
acetoacetate	–	–	50	143

appropriate chain length of not more than 7 C-atoms function as potent effectors of CO_2 fixation. A collection of more than 20 of such substances is presented in Table I in order to deduce structure-activity relationships and to clarify the specificity of the effector binding site.

All these effectors are distinguished by the following characteristic properties:

a) They enhance the carboxylation reaction at relatively low concentration, when the enzyme is in a limited activation state and inhibit this process at higher concentrations, as is demonstrated in Fig. 2 for some of these agents. The inhibition occurs competitively to the substrate RuP$_2$. All the diagrams in Fig. 2 are characterized by an effector maximum that results from a superposition of the stimulation and inhibition effects induced by these compounds. These experiments were performed at 0.1 mM NaHCO$_3$ and 20 mM MgCl$_2$.

b) All the effectors in Table I which equilibrate rapidly with the enzyme decrease K_{act} (CO_2), *i.e.* they stabilize the active ternary complex enzyme · $^A CO_2$ · Mg^{2+} and support CO_2 fixation. The effector concentrations for the determination of K_{act} (CO_2) refer to the maxima of effector activity profiles, as shown in Fig. 2. The stabilization of the reactive carbamate is also obvious from Fig. 3 which demonstrates that the dissociation of this intermediate is retarded under such conditions. In order to compare the full regulatory capacity of such effectors, the dilution method [12, 14] was applied. The concept of this technique is to saturate the effector binding sites and thus to develop the maximal activation effect. Under these conditions,
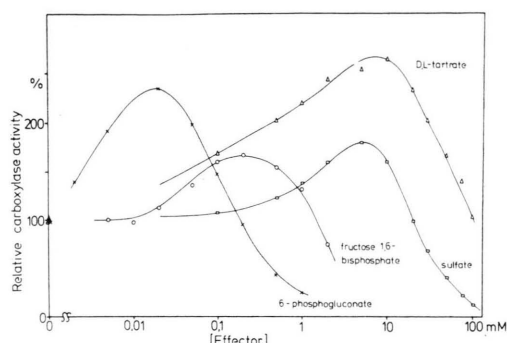
Fig. 2. Relative carboxylase activity as a function of the effector concentration (assay method a). The reference without effector (100%) corresponds to a carboxylation rate of 0.18 μmol mg protein^{-1} min^{-1} (0.18 mg protein per assay).
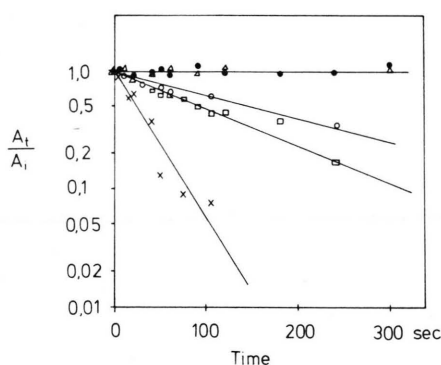

Fig. 3. Effector dependent modification of the dissociation of the activated ternary complex carboxylase · ^{A}CO$_2$ · Mg^{2+} at 10 °C. The enzyme was first activated for 1 h in 0.1 M bicine-KOH-buffer, pH = 8.0 containing 10 mM NaHCO$_3$, 10 mM MgCl$_2$, 0.1 mM EDTA and 1 mM DTE. 10 μl aliquots of the reaction mixtures were diluted 100 fold into 990 μl CO$_2$-depleted 0.1 M bicine-buffer, pH = 8.0, 0.1 mM EDTA and 1 mM DTE. The dilution medium was either free of effector (x) or contained 1 μM (○); 20 μM (●) and 200 μM (△) 6-phosphogluconate or 20% (v/v) acetone (□). At the indicated times after dilution of the enzyme CO$_2$ fixation was started by addition of the reaction components (10 mM NaHCO$_3$, 10 mM MgCl$_2$ and 0.5 mM RuP$_2$) and assayed for 30 s. The ratio of the activity A_t at time t and the initial activity A_i at $t = 0$ was plotted half-logarithmically as a function of the length of the time intervals between dilution and assay of the enzyme.

however, the enzyme is completely inhibited and therefore catalytically incompetent, because Badger and Lorimer [12] have shown that such effectors bind exclusively at the reaction centers of the carboxylase within the RuP$_2$ binding domain. For the detection of the activation state of the enzyme the reaction mixtures have to be diluted sufficiently.

The experiments demonstrated in Fig. 4 and Table I were performed, as follows: Samples of the spinach enzyme were saturated with effectors by preincubation at high inhibitory modifier concentrations and low CO$_2$ activation conditions (0.1 mM NaHCO$_3$; 10 mM MgCl$_2$). Afterwards they were diluted 200 fold into the assay mixture. By this procedure the effector concentrations in the assay were reduced to levels at which most of these compounds do not affect CO$_2$ fixation appreciably. The assay time was reduced as far as possible (15 s) to establish that the activation state of the enzyme reached during preincubation was not changed significantly during the assay. As demonstrated for 6-phosphogluconate in Fig. 4 the effector activity increases under such conditions up to high effector concentrations which normally inhibit the carboxylase reaction completely (see Fig. 2). The maximal activation levels obtained by the dilution method are summarized in Table I. If RuP$_2$ and effectors compete for a common binding site at the enzyme, one should expect that also RuP$_2$ displays a stabilizing effect on the reactive carbamate complex which is the activated form of the Rubisco. To test this assumption we added RuP$_2$ in variable concentration to the activation mixture. Under such experimental conditions CO$_2$ fixation proceeded already in the preincubation medium, but the substrate pool was not completely consumed during the activation period. Our results show that also RuP$_2$ induces a decrease of K_{act} (CO$_2$) similar to the effector anions. At initial concentrations of 0.04 and 0.2 mM RuP$_2$ in the activa-

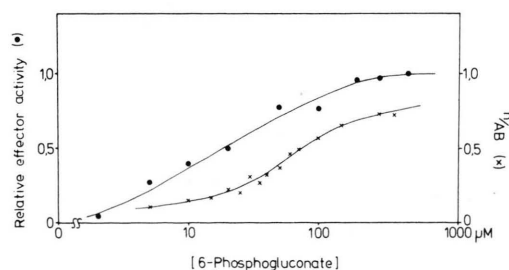
Fig. 4. Relative effector activity of 6-phosphogluconate (●) and [^{14}C-6]phosphogluconate binding (x) to the spinach carboxylase as a function of the effector concentration. AB is one protomer of the carboxylase which consists of one large and one small subunit A and B. n/AB indicates the number of effectors bound per protomer AB. Assay method b; the activation mixtures were diluted 200 fold with the assay mixture. The maximum effector activity of 6-phosphogluconate was 408%. [^{14}C-6]phosphogluconate binding was detected by the ultrafiltration method.

tion mixture K_{act} (CO_2) of 64 and 20 μM, respectively, were obtained compared to K_{act} (CO_2) = 71 μM in the absence of a modifier.

2) Correlation between effector activity and effector binding

In order to clarify the question, how ribulose 1,5-bisphosphate carboxylases/oxygenases remain catalytically competent in the presence of effectors that interact at the reaction centers we correlated activity measurements with binding studies.

In Fig. 3 measurements of the dissociation kinetics of the reactive carbamate of the carboxylase have been performed. The enzyme was activated in the presence of each 10 mM $NaHCO_3$ and $MgCl_2$ for 30 min at 25 °C. The activation mixture was then diluted 100 fold into CO_2-depleted buffer at 10 °C. Under these conditions the previously formed active ternary complex decomposes in a first order kinetics with a half life time of $t_{1/2} = 28$ s. This process is retarded already at very low 6-phosphogluconate concentrations as 1 μM ($t_{1/2} = 145$ s). In the presence of 20 μM 6-phosphogluconate which corresponds to its effector maximum at low CO_2-concentrations, when assayed by method a (see Fig. 2), the reactive carboxylase-carbamate complex is completely stabilized. Under such conditions only small portions of the reaction centers are occupied by the modifier (6% at 1 μM and 16% at 20 μM resp.). Obviously relatively low, non-saturating effector concentrations induce high activation levels of the carboxylase. This conclusion is supported by the experiments shown in Fig. 4 which indicate that the pofile for the activation of the enzyme is shifted to lower modulator concentrations compared to effector binding. At 20 μM 6-phosphogluconate a nearly threefold increase of the carboxylation rate is observed which is more than half of the total stimulation effect induced by this effector. Similar results have been obtained for other effectors.

These data are also consistent with the information derived from competition experiments with combinations of two effectors. In Fig. 5 the action of fructose-1,6-bisphosphate on the binding and activity of 6-phosphogluconate is shown. In a series of experiments fructose 1,6-bisphosphate in increasing concentration was added to the preincubation mixture along with 20 μM 6-phosphogluconate. Also in this case the stimulation effect of 6-phosphogluconate is more strongly affected by the
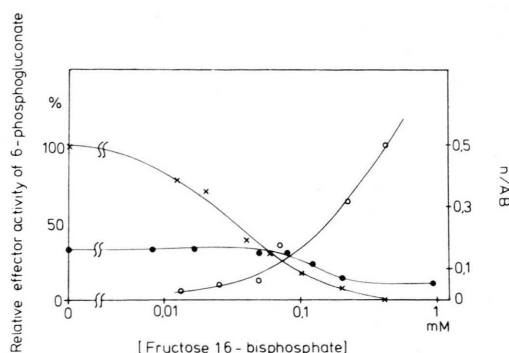

Fig. 5. Relative effector activity of 6-phosphogluconate (×) and [^{14}C-6]phosphogluconate binding (●) as a function of the fructose 1,6-bisphosphate concentration as well as the binding profile of fructose 1,6-bisphosphate (○). All samples contained 20 μM 6-phosphogluconate. The effector activity of this agent was measured by assay method a. The stimulation effect in the absence of fructose 1,6-bisphosphate was 278%. The carboxylation rate under these conditions was 0.1 μmol · mg protein^{-1} · min^{-1} (0.22 mg protein per assay). Both effectors were added simultaneously to the preincubation mixtures. Effector binding was detected by the ultrafiltration technique.

second modifier than its binding. For example when only 10% of the effector binding sites are occupied by fructose 1,6-bisphosphate, a loss of 70% of the effector activity of 6-phosphogluconate is observed. Obviously fructose 1,6-bisphosphate interacts more rapidly with most of the free effector binding sites than 6-phosphogluconate and displays its specific activation of the enzyme. This conclusion is consistent with preliminary results of the kinetic analysis of effector binding using rapid detection techniques (J. Vater and N. Erdin unpublished). Only at higher concentrations competition between both effectors occurs which leads to a displacement of bound 6-phosphogluconate.

III. The effect of the polarity of the reaction medium on catalysis and regulation of CO_2 fixation

In Figs. 6a and b it is demonstrated that the effects of some organic solvents on the CO_2 fixation process are similar to the action of dianionic effectors (see Fig. 2). Solvents, as methanol, ethanol or acetone with dielectric constants between 20−40, stimulate the carboxylation reaction at relatively low concentrations (1−15%, v/v), if assayed at limiting bicarbonate concentrations (Fig. 6a). These compounds stabilize the active ternary complex enzyme · $^{A}CO_2$ · Mg^{2+}, as is apparent from the
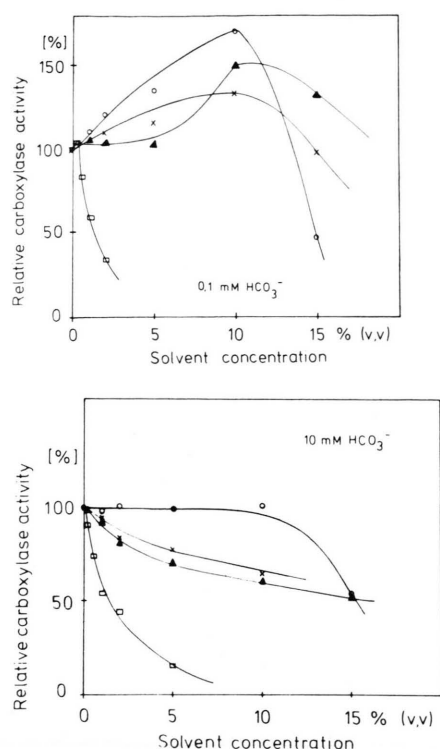
Fig. 6. Relative carboxylase activity as a function of the concentration of various organic solvents in the reaction mixtures. For the measurement of CO_2 fixation assay method a was used in the presence of either a) limiting (0.1 mM) or b) saturating (10 mM) bicarbonate concentrations. (×) methanol; (○) ethanol; (▲) acetone; (□) dioxane. The carboxylation rates in the absence of solvents were 0.21 and 1.34 μmol mg protein^{-1} min^{-1} at 0.1 mM and 10 mM NaHCO$_3$, respectively (0.28 mg protein per assay).

kinetic experiment in Fig. 3. At 10 °C the half life time $t_{1/2} = 28$ s for the dissociation of the reactive carbamate is increased more than three-fold ($t_{1/2} = 90$ s), if 20% acetone are added to the preincubation medium. This is also obvious from a decrease of the K_{act} (CO_2) in the presence of such solvents. When

8% (v/v) of methanol, ethanol or acetone were added to the reaction mixture, K_{act} (CO_2) data of 54; 41 and 38 μM, respectively, have been measured compared to K_{act} (CO_2) = 71 μM without addition of solvent.

The stimulating effects of such solvents are abolished by the addition of dianionic effectors, as 20 μM 6-phosphogluconate, for example. At solvent concentrations higher than 10−15% (v/v) CO_2 fixation is inhibited and the enzyme tends to precipitate. Also at saturating bicarbonate and Mg^{2+} concentrations which transform the carboxylase to the activated state, the solvent induced enhancement of the carboxylation rate disappears and only the inhibition phase is observed (Fig. 6b).

If solvents with low dielectric constants, as, for example, dioxane or tetrahydrofurane were applied, only weak stimulation effects have been found at concentrations between 0.03−1% (v/v), if assayed at limiting bicarbonate concentrations. In this case the inhibition predominates, as is shown in Figs. 6a and b for dioxane.

IV) An analysis of functional groups involved in the binding of anionic effectors

Several authors [20−24] have demonstrated that specific cysteinyl, lysyl, arginyl and histidyl residues are essential for the catalysis and regulation of CO_2 fixation. In order to characterize the binding sites for dianionic effectors of the spinach carboxylase we examined the functional groups involved in the binding and activity of such compounds.

In Table II the effects of a) N-methyl-maleimide (NMM) as a thiol inhibitor, b) pyridoxal 5′-phosphate (PLP), a reagent for amino groups of reactive lysine residues and c) phenylglyoxal (PGO) as an arginine specific modifier were investigated.

Table II. The effect of some group specific reagents on CO_2-fixation as well as on the effector activity and binding of 6-phosphogluconate. Assays and detection of effector binding were performed as indicated under Methods. The 6-phosphogluconate concentration in the reaction mixtures with effector (experiments b and c) was 20 μM. The carboxylation rate in the absence of inhibitor (100%) was 0.15 μmol mg protein^{-1} min^{-1} (0.18 mg protein per assay).

Reagent	a) CO_2-fixation [%]	b) Effector activity of 6-phosphogluconate [%]	c) Binding of 6-phosphogluconate [%]
—	100	100	100
10 mM Phenylglyoxal	8.5	620	100
0.5 mM N-methylmaleimide	10	154	100
0.5 mM Pyridoxal 5′-phosphate	20	18	55

The results obtained show that under reaction conditions, when CO_2 fixation is reduced to 10−20% of its original rate, the effector binding of 6-phosphogluconate is not impaired by NMM and PGO. The activity of this effector is even enhanced in the presence of these inhibitors. The reason for this increase remains to be clarified. In contrast lysine specific reagents, as PLP or 2,4,6-trinitrobenzenesulfonate efficiently reduce CO_2 fixation as well as binding and activity of 6-phosphogluconate. Similar results have been achieved for some other effectors from the collection in Table I.

Discussion

The *in vivo* levels of CO_2 and Mg^{2+} in the stroma of chloroplasts (10 μM CO_2, 5−10 mM Mg^{2+} at pH = 8) cannot account for the high CO_2 fixation rates observed under physiological conditions. The Rubisco would be retained mainly in its inactive form. Efforts have been made to clarify this discrepancy.

Several authors [4, 5, 7−12, 14] have demonstrated that the carboxylation and oxygenation of RuP_2 can be modulated *in vitro* by certain anionic chloroplast metabolites. A few of them, as for example, NADPH or 6-phosphogluconate, stimulate these processes at suboptimal CO_2 and Mg^{2+} concentrations and may, therefore, play an important role in the *in vivo* regulation of photosynthetic CO_2 fixation.

In this publication we want to emphasize that the effects of such modifiers on the catalysis and regulation of the carboxylation process can be classified depending on their anionic structure. Our results demonstrate a fundamental difference in the mode of action of monovalent anions and modulators with a dianion structure.

Monovalent anions predominantly inhibit CO_2 fixation and the formation of the active ternary complex carboxylase $\cdot\ ^ACO_2 \cdot Mg^{2+}$, while the activity and binding of NADPH and effector sugar phosphates is impaired only at appreciably higher concentrations.

Though the effects of such anions studied in this publication occur under nonphysiological conditions they reflect characteristic differences in the reactivity of functional elements involved in the catalysis and regulation of CO_2 fixation.

In contrast bivalent anions, like phosphate and sulfate as well as some chloroplast metabolites with a dianionic structure (6-phosphogluconate, fructose, 1,6-bisphosphate etc.) and structurally related compounds increase the affinity of the carboxylase for the activator CO_2 and stabilize the active ternary complex. Recent investigations [12, 14] characterize such modifiers as RuP_2 like, active site directed effectors which support the CO_2 activation of the enzyme and stimulate CO_2 fixation. Our results are consistent with this model on the mode of action of these agents. The main objection to such a mechanism that remains to be clarified is, how an enzyme can be catalytically competent under such conditions. The subject of this publication is to contribute to solution of this central question and to investigate characteristic features of the effector binding sites.

The specificity of these sites has been investigated performing structure-activity-studies with a collection of more than 20 of such effectors. The activation constant K_{act} (CO_2) and the maximal effector activity, as determined by the dilution method, were taken as indicators for the effectiveness of these compounds. These data correlate fairly well. The essential functional elements of such compounds are two negatively charged groups, as, for example, carboxyl or phosphate residues which may be linked by maximally 6−7 C-atoms. Such effectors are structural analogues of the substrate RuP_2. Our data present further evidence that RuP_2 in addition to its role in the catalysis of CO_2 fixation may function as an activator of the Rubisco under certain reaction conditions.

The data in Table I reveal a broad specificity of the effector binding sites. The structural elements of RuP_2 obviously can be reduced to its negatively charged terminal groups, because phosphate still is a strong stimulator of the carboxylase.

The effector activity of dicarboxylic acids increases starting from oxalate up to glutarate which is composed of 5 C-atoms, similar to RuP_2. Our studies show that anions of higher dicarboxylic acids, as for example adipic-, pimelic- or suberic acid do not induce a stimulation effect anymore.

Hydroxy- and α-ketodicarboxylic acids which are structurally more closely related to RuP_2 usually are more potent effectors than the unsubstituted compounds.

Our experiments with group specific reagents demonstrate that essential amino groups are involved in binding and activity of dianionic effectors. In contrast to the catalysis of CO_2 fixation and

RuP$_2$ binding [20, 21, 23] the participation of cysteines and arginines could not be detected. These features point to the existence of two positively charged countergroups at the effector binding sites which interact with the negatively charged terminal groups of the dianionic effector molecules. It remains to be clarified, whether or not these countergroups are identical with two reactive lysines that are essential for the binding of the substrate RuP$_2$ at the reaction centers of the carboxylase identified by Hartman and his colleagues [21, 22]. RuP$_2$ and structurally similar effectors, as 6-phosphogluconate which are composed of 5−6 C-atoms apparently span the distance between the reactive amino groups. It has to be established by quantitative binding studies, if in the case of smaller effectors, as bivalent inorganic anions or short dicarboxylic acids, two molecules are bound to the effector sites.

Catalysis and regulation of CO_2 fixation also differ in their dependence on the polarity of the reaction medium. If this parameter is decreased by the addition of organic solvents, as methanol, ethanol or acetone, in a concentration range between 1−15% (v/v) a modification of the regulation centers of the carboxylase occurs which enhances the affinity of the enzyme for the activator-CO_2 and stabilizes the reactive carbamate. This is demonstrated in Figs. 3 and 6a. On the other hand the catalytic process is progressively inhibited with increasing solvent content of the reaction medium (see Figs. 6a and b). The inhibition predominates in the presence of solvents with low dielectric constants, as dioxane or tetrahydrofurane.

The effect of such solvents is obviously similar to the action of dianionic effectors. The stimulation of the CO_2 fixation by organic solvents is abolished in the presence of such compounds. We therefore infer, that as a consequence of effector binding to the raction centers a decrease in the polarity is induced at the regulatory sites of the enzyme which modifies the properties of the active lysine for the binding of the $^{A}CO_2$ presumably by a change of the pK of this group. This modification may be accomplished by conformational changes of the enzyme that occur on effector binding, as demonstrated fluorimetrically in previous publications [10, 19]. This conclusion is supported by the findings of Hatch and Jensen [11] that anionic effectors, as 6-phosphogluconate, affect the pH profile of CO_2 activation shifting it towards lower pH values.

Our results suggest that the central problem concerning the catalytical competence of D-ribulose 1,5-biphosphate carboxylases/oxygenases in the presence of active site directed dianionic effectors can be solved kinetically.

The CO_2 activation of the carboxylase proceeds slowly over at least 10−15 min at 25 °C. Also the dissociation of the enzyme · $^{A}CO_2$·Mg^{2+} complex with a half life time of 20−50 s is a rather slow process compared to the rapid equilibration of the enzyme with the dianionic effectors listed in Table I.

If one assumes that the rate constants for the dissociation of the carboxylase-effector-complexes are essentially higher than those for the formation of these intermediates the residence time of an effector at the reaction center would be short compared to the lifetime of the activated state of the carboxylase. Under such conditions a relatively low effector concentration is sufficient to interact rapidly with a considerable quantity of enzyme molecules inducing the active conformation. In this way most of the reaction centers remain catalytically competent and the enzyme activity is stimulated. At higher effector concentrations increasing occupation of the reaction centers by effector molecules leads to inhibition even with the enzyme being in a state of higher activation.

This kinetic interpretation of the catalytical competence of the Rubisco under such conditions is supported by the results of our binding studies and activity measurements with effectors. The experiments in Figs. 3−5 demonstrate that low, nonsaturating effector-concentrations induce high activation levels of the carboxylase.

The experimental material so far available points to an unusual mechanism of the complex regulatory processes involved in photosynthetic CO_2 fixation which are effective essentially at limiting substrate and effector concentrations. Obviously the homotropic stimulation effect which is induced by the activator CO_2 can be regulated heterotropically by active site directed chloroplast metabolites. The physiological relevance of these phenomena needs, however, clarification in more detail.

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