Activation of ribulose 1,5-bisphosphate carboxylase by Ca^{2+}

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Purified RuBP carboxylase requires activation by reaction with CO_2 and a divalent metal ion. Mg^{2+} is the most effective metal ion, and is probably involved in activation in vivo. Ca^{2+} is reported not to be an activator. Several oxyanions, including phosphate esters, are effectors of activation of RuBP carboxylase by Co_2 and Mg^{2+}. It is now shown that Ca^{2+} is an effective activator of RuBP carboxylase and that PO_4^{3-}, FBP, NADPH and 6-phosphogluconate are effectors of this activation. The ratio of oxygenase to carboxylase activity of enzyme activated with Ca^{2+} is similar to that for the enzyme activated with Mg^{2+}.

RuBP carboxylase *Activation by Ca^{2+}* *6-Phosphogluconate* *Oxyanion effector*

1. INTRODUCTION

To catalyse the carboxylation and oxygenation of RuBP, purified RuBP carboxylase (EC 4.1.1.39) requires activation by CO_2 and a divalent metal ion [1]. The purified spinach enzyme can be reversibly activated in a few minutes at 25°C; maximum activity is achieved at pH 8.2 in the presence of 10 mM sodium bicarbonate, to provide the CO_2, and 20 mM $MgCl_2$ [2]. Maximum activity can be achieved at lower concentrations of CO_2 and Mg^{2+} if certain effectors of activation are added, for example NADPH and 6-PG. Such effectors appear to stabilize a carbamate–Mg^{2+} complex formed when the activating CO_2 reacts with the ϵ-amino group of a lysine residue of the large subunit polypeptides. The mechanism involves the effectors interacting with the RuBP binding site [3,4].

Purified RuBP carboxylase from wheat leaves differs in properties from the spinach enzyme in that it exists in two forms [5,6]. One form is activated very slowly during incubation with CO_2 and Mg^{2+} ($t_{0.5}$ ~1 h at 25°C). After this activation the protein is in a second form that deactivates and reactivates in a few minutes upon removal and re-addition of CO_2 and Mg^{2+}. Effectors of activation of the spinach enzyme also facilitate activation of the wheat enzyme [6].

RuBP carboxylase was activated also by Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} [7–11] but none of these ions was as effective as Mg^{2+} and all changed the catalytic activity of oxygenase relative to carboxylase when compared to the Mg^{2+}-activated enzyme. Two reports state that Ca^{2+} did not activate the enzyme [8,10]. However, we show that Ca^{2+} can activate RuBP carboxylase from wheat.

Abbreviations: 6-PG, 6-phospho-D-gluconic acid; FBP, D-fructose 1,6-bisphosphate; RuBP, D-ribulose 1,5-bisphosphate

2. MATERIALS AND METHODS

2.1. *RuBP carboxylase from wheat leaves*

RuBP carboxylase was purified from wheat leaves as in [6] except that 5 mM bicine (pH 8.0) was used for equilibration of the Sephadex G-25 column instead of HEPES. Elemental analysis using argon emission spectrometry showed the presence of 0.14 Mg atom/active site.

2.2. *Activation*

Freeze-dried enzyme, 7–14 mg/ml, was dissolv-

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ed in the appropriate solution of Ca^{2+} or Mg^{2+} in 0.1 M or 0.09 M bicine buffer (pH 8.2) with or without an effector, and in the presence of 5 mM $NaHCO_3$. The solutions were incubated at 25 or 40°C.

2.3. *Measurement of carboxylase activity*

Carboxylase activity was measured by adding 10–25 μl activated or partly activated enzyme, 140–280 μg protein, to O_2-free 0.1 M bicine (pH 8.2), 5 mM $NaH^{14}CO_3$ and 0.33 mM RuBP, with or without either 5 mM $MgCl_2$ or 15 mM $CaCl_2$, in the stoppered vessel of a Hansatech (Kings Lynn, Norfolk) oxygen electrode at 25°C. The total volume of the reaction mixture was 1 ml. Reaction was stopped by injecting 50 or 100 μl samples into 100 μl 4 N formic acid. The acidified samples were evaporated to dryness overnight in an oven at 60°C in vials for liquid scintillation counting. The amount of ^{14}C in the non-volatile residue was measured.

2.4. *Measurement of oxygenase activity*

Reaction mixtures were similar to those for measurement of carboxylase activity except that the buffer used was equilibrated with CO_2-free air at 25°C, to provide 260 μM O_2, and the $NaH^{14}CO_3$ was omitted. Activity was measured from the initial rate of oxygen uptake.

2.5. *Measurement of carboxylase and oxygenase in the same reaction mixture*

Reaction mixtures, final volume 1.0 ml in an oxygen electrode vessel, contained 0.945 ml 0.1 M bicine equilibrated with air (pH 8.2) containing 13 μg/ml of freeze-dried carbonic anhydrase, 0.01 ml 40 mM RuBP, 0.01 or 0.02 ml 0.1 M $NaH^{14}CO_3$ (1 μCi/μmol) and where necessary, 0.01 ml H_2O. Reaction was started by adding 0.025 ml of a solution of activated protein and stopped after 40 s by the addition of 0.05 ml 10 M formic acid. During the reaction the oxygen concentration was recorded at 10 times the normal sensitivity with 90% of the signal from the electrode backed off. The oxygenase activity was calculated from the total oxygen taken up in the 40 s of reaction time (and not from the initial rate of uptake). The carboxylase activity was measured from the acid non-volatile radioactivity in a sample of the acidified reaction mixture.

2.6. *Materials*

$NaHCO_3$, $CaCl_2 \cdot 6\,H_2O$ and $MgCl_2 \cdot 6\,H_2O$ were AR grade chemicals from Fisons (UK). Carbonate-free NaOH was from BDH Chemicals (Poole). Bicine (*N,N*-bis[2-Hydroxyethyl]glycine), carbonic anhydrase, RuBP and 6-PG were from Sigma Chemicals (St Louis MO). $NaH^{14}CO_3$ was from Amersham International (Bucks).

3. RESULTS

Table 1 shows that Ca^{2+} and Mg^{2+} both activate RuBP carboxylase at 40°C. The extent of activa-

Table 1

Activation of RuBP carboxylase (nmol.mg protein^{-1}.min^{-1}) by Mg^{2+} and Ca^{2+} in the presence and absence of effectors of activation

Divalent ion at 5 mM	Effector (mM)	Divalent metal ion added for assay		
		None	5 mM Mg^{2+}	15 mM Ca^{2+}
Mg^{2+}	None	420	480	520
Ca^{2+}	None	40	170	40
Mg^{2+}	6-PG (2)	870	1080	950
Ca^{2+}	6-PG (2)	480	770	610
Mg^{2+}	NADPH (2)	660	1070	800
Ca^{2+}	NADPH (2)	270	580	310
Mg^{2+}	NADPH (5)	910	1090	840
Ca^{2+}	NADPH (5)	250	560	310
Mg^{2+}	FBP (2)	360	700	590
Ca^{2+}	FBP (2)	70	240	130
Mg^{2+}	FBP (5)	320	660	490
Ca^{2+}	FBP (5)	50	190	70
Mg^{2+}	PO_4^{3-} (5)	810	1130	810
Ca^{2+}	PO_4^{3-} (5)	140	310	180

Freeze-dried enzyme was dissolved (9 mg/ml) in 0.09 M bicine (pH 8.2) containing 10 mM $NaHCO_3$ with metal ions and effectors as detailed below. The solutions were incubated at 40°C for 40 min. Carboxylase activities were estimated in 0.01 ml samples as in section 2.3. Each reaction mixture contained also 50 μM Mg^{2+} or Ca^{2+} from the enzyme solution. Results shown are means of at least two measurements

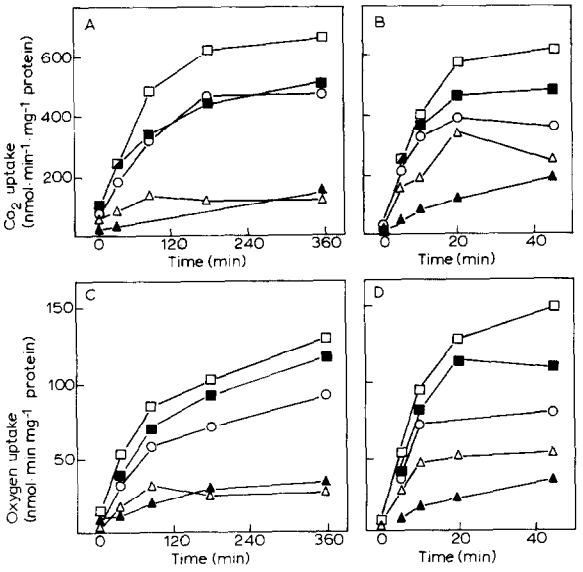

Fig.1. Activation of RuBP carboxylase measured by carboxylation (A,B) and oxygenation (C,D) at 25°C (A,C) or 40°C (B,D). The enzyme was activated by: 5 mM $NaHCO_3$, 20 mM $MgCl_2$ (—○—); 5 mM $NaHCO_3$, 5 mM $MgCl_2$ (—△—); 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 2 mM 6-PG (—□—); 5 mM $NaHCO_3$, 5 mM $CaCl_2$ (—▲—); 5 mM $NaHCO_3$, 5 mM $CaCl_2$, 2 mM 6-PG (—■—). Activity was measured at 25°C in a total volume of 1.0 ml containing 0.095 M bicine (pH 8.2), 0.33 mM RuBP and 140 μg protein. Oxygenase was measured, in the absence of added CO_2, in buffer saturated with air (260 μM O_2). Carboxylase was measured in O_2-free buffer, in the presence of 5 mM $NaH^{14}CO_3$, from the ^{14}C present in acid-stable products after 30 s.

tion by Ca^{2+}, like activation by Mg^{2+}, was increased by the addition of certain effectors in the order 6-PG > NADPH > PO_4^{3-} > FBP. The amounts of activity measured were increased if 5 mM Mg^{2+} or 15 mM Ca^{2+} were present during assay. The added divalent ions probably prevented breakdown of the enzyme carbamate metal ion complex (deactivation) rather than directly affecting catalysis, but it is possible that both functions were involved.

Fig.1 confirms that Ca^{2+} alone, and especially in the presence of 6-PG, activates RuBP carboxylase; it also shows that activation takes place at both 25°C and 40°C, and that the oxygenase function is also activated. The time required for activation by Ca^{2+} was similar to that required for activation by Mg^{2+}. In the absence of effectors, activation by 5 mM Ca^{2+} was equal at 25°C but less at 40°C than activation by 5 mM Mg^{2+}. The activation achieved with Ca^{2+}, in the presence of 5 mM $NaHCO_3$ and 2 mM 6-PG, was equal to (25°C) or greater than (40°C) activation by 10 mM $NaHCO_3$ with 20 mM $MgCl_2$, concentrations known to be optimal for activation of the wheat enzyme in the absence of effectors. Activation was less with 5 mM Ca^{2+} than with 5 mM Mg^{2+} in the presence of 5 mM $NaHCO_3$ and 2 mM 6-PG. Comparison of fig.1C with fig.1A shows that the oxygenase activity following activation with Ca^{2+} and 6-PG correlated better with that achieved with Mg^{2+} and 6-PG than did the corresponding carboxylase activities. This could suggest a difference in the

Table 2

Carboxylase (v_c) and oxygenase (v_o) activities (nmol.min^{-1}.mg protein^{-1}) and v_c/v_o ratio for RuBP carboxylase from wheat leaves activated by Ca^{2+} compared to Mg^{2+}

Activating ion	Measurements	$NaH^{14}CO_3$ (mM)	v_c	v_o	v_c/v_o
Mg^{2+}	7	2.125	466 ± 27	56.4 ± 2.9	8.26 ± 0.20
Ca^{2+}	6	2.125	302 ± 15	36.4 ± 1.7	8.30 ± 0.22
Mg^{2+}	3	1.125	370 ± 10	78.1 ± 2.9	4.74 ± 0.11
Ca^{2+}	3	1.125	234 ± 10	50.4 ± 1.2	4.64 ± 0.14

The freeze-dried enzyme was dissolved, 15.15 mg protein/ml, in 0.1 M bicine (pH 8.2) containing 5 mM 6-PG, 5 mM $NaHCO_3$ and either 5 mM Ca^{2+} or 5 mM Mg^{2+}. The solutions were kept at 40°C for 40 min and stored for 1 h at 25°C prior to measurement of v_c and v_o as in section 2.5. Final concentrations present were: 0.095 M bicine (pH 8.2), 0.4 mM RuBP, 260 μM O_2, either 2.125 or 1.125 mM $NaH^{14}CO_3$, 0.125 mM 6-PG and 0.125 mM Ca^{2+} or Mg^{2+}. Values shown are means ± standard deviation

relative activity of the two enzymes as carboxylases compared to oxygenases. However, in this experiment oxygenase and carboxylase were estimated in separate reaction mixtures where the rates of deactivation were probably different.

Table 2 shows that when carboxylase and oxygenase activities were measured in the same reaction mixture by $^{14}CO_2$ and O_2 uptake in 40 s, their relationship (v_c/v_o) was not significantly different for enzyme activated by Mg^{2+} in the presence of 6-PG and enzyme activated with Ca^{2+} under similar conditions. This conclusion was valid with each of two concentrations of $NaHCO_3$ providing either 17.5 or 9.3 μM CO_2 in solution with 260 μM O_2. The ratios of v_c/v_o obtained were 8.3 and 4.7, respectively, and were in agreement with values obtained for the purified wheat enzyme under similar conditions in other studies (not shown).

4. DISCUSSION

Ca^{2+} in the presence of CO_2 was reported not to activate RuBP carboxylase from soybean [10] or spinach [8] but we report a considerable activation of the wheat enzyme by this ion. Under our conditions, Ca^{2+} also activated RuBP carboxylases from maize, rye-grass and spinach (not shown). The failure [8] to activate the spinach enzyme was probably because [Ca^{2+}] used was too low. However, the main factor involved may be providing conditions in the medium used for activation that keep Ca^{2+} in solution by delaying or preventing precipitation. In particular, $CaCO_3$ is of extremely low solubility (<0.2 mM).

Wheat enzyme activated in the presence of 6-PG showed the same ratio of activity of carboxylase to oxygenase whether activated with Ca^{2+} or by Mg^{2+} as divalent metal ion. It is well established for enzyme activated with Mn^{2+} without an effector, that carboxylase activity is much decreased relative to oxygenase activity compared to the Mg^{2+} activated enzyme [7,9,11]. The ratio v_c/v_o was similar whether the enzyme was activated with Mn^{2+} in the presence of 6-PG or with Mn^{2+} alone (not shown). Further comparisons of enzyme activated with Ca^{2+}, Mg^{2+} or Mn^{2+} may help to show whether divalent ions are required for catalysis or merely stabilize the carbamate formed between a lysyl residue on the enzyme and the activating CO_2 [12]. The cation may also be involved in catalysis either by stabilizing the intermediate of RuBP or by direct interaction with CO_2 or O_2 [11].

There are considerable quantities of Ca^{2+} in the chloroplast [13,14] but this is mostly bound to the thylakoid membranes in the light. In the dark, acidification may lead to a release of bound Ca^{2+}. Also in the dark the effector of activation, 6-PG, would increase [15]. Since this effector is active both with Mg^{2+} and with Ca^{2+}, activation by Ca^{2+} would not be favoured more than Mg^{2+}. However, the [Mg^{2+}] in the chloroplast stroma is less in darkness than in the light [16,17] so an increased proportion of RuBP carboxylase may become associated with Ca^{2+}. Since there is some evidence that RuBP carboxylase activity must be low in darkness [18], further studies should be made of the properties of the enzyme in the presence of Ca^{2+} to see whether it is more rapidly and reversibly deactivated in the presence of particular metabolites associated with dark metabolism than the corresponding enzyme activated with Mg^{2+}.

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