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Activation of Rubisco from non-green algae

I Saska, SM Whitney, HJ Kane, TJ Andrews

Molecular Plant Physiology Group, Research School of Biological Sciences, The Australian National University, PO Box 475, Canberra, ACT, 2601, Australia. saska@rsbs.anu.edu.au

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Introduction

The CO_2 fixing enzyme, D-ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), must be activated prior to substrate binding in order to function as a catalyst. Activation involves the binding of two co-factors at the active site; a molecule of CO_2 that is distinct from the substrate of the carboxylase reaction, and a Mg^{2+}ion. CO_2 attaches to the ε-amino group of a conserved lysine residue at the active site, forming a carbamate that, in turn, completes an anionic co-ordination site for the Mg^{2+}. Facilitated by access to a resonant structure with a full negative charge on both of its terminal oxygen atoms, this metal-coordinated carbamate appears to be the general base that carries out several proton abstractions essential to the catalytic sequence. This function is without precedent elsewhere in biology.

In the absence of substrate, formation of the activated enzyme-CO_2-Mg^{2+} (ECM) complex reaches an equilibrium governed by the CO_2 (C) and Mg^{2+} (M) concentrations:

$$E + CO_2 \overset{K_c}{\rightleftharpoons} EC + Mg^{2+} \overset{K_{Mg}}{\rightleftharpoons} ECM$$

The product of the dissociation constants for CO_2 and Mg^{2+}, K_CK_{Mg}, therefore dictates the extent of activation of Rubisco at particular CO_2 and Mg^{2+} concentrations.

Recently it has been found that certain non-green algae possess Rubiscos with more efficient kinetic properties than those of higher-plant Rubiscos. Consequently, attention has been focused on these Rubiscos as potential candidates for transfection into higher plants. Nothing, however, is known about the activation properties of non-green algal Rubiscos and whether they would be suitable to the conditions prevailing in higher-plant chloroplasts. The aim of this work is the elucidation of the carbamylation equilibrium of Rubisco from non-green algae.

The finding (Pierce, Tolbert et al. 1980) that the transition state analogue 2'-carboxy-D-arabinitol 1,5-bisphosphate (CABP) binds to ECM much more tightly ($K_d \sim 10^{11}$ M) than it does to the unactivated form of the enzyme (E and/or EC) facilitated the development of more direct approaches to determining ECM concentration. Before this, levels of ECM were inferred from enzyme activity measured rapidly after incubation at various Mg^{2+} and CO_2 concentrations. In combination with $^{14}CO_2$, CABP can be used to 'trap' activated E^{14}CM complexes that have been equilibrated at defined NaH$^{14}CO_3$ and Mg^{2+} concentrations. The radioactivity bound as the carbamate can then be isolated by gel filtration and quantified. Alternatively, ^{14}C-CABP can be allowed to bind to enzyme that has been pre-equilibrated at certain CO_2 and Mg^{2+} concentrations. While the [^{14}C]-CABP will bind to both carbamylated and uncarbamylated forms of the enzyme, the subsequent addition of an excess of unlabelled

CABP displaces the label from weakly bound E-[^{14}C]-CABP complexes, so that only the ECM complexed sites remain radioactively labelled and, following gel filtration, quantifiable.

Materials and Methods

Preparation of Rubisco. Higher-plant Rubisco was purified from spinach (Spinacea oleracea L.) leaves according to Edmondson et. al. (Edmondson, Badger et al. 1990a) omitting the final gel filtration step. Before use, it was activated in 10 mM $NaHCO_3$, 10 mM $MgCl_2$ at 50°C for 15min and then decarbamylated by dialysing in 20-100 mM EPPS-NaOH, pH 8.0, 0.1 mM EDTA, 1 mM DTT with N_2 sparging, for ≥4 hrs. To maintain the enzyme in a decarbamylated state, it was immediately transferred to a N_2 flushed septum-sealed vial.

Galdieria sulphuraria cultures, grown in *Cyanidium* medium (Allen 1959) in 20 L glass containers at 32°C (±2°) under constant illumination (480-660 µmol quanta m^{-2} s^{-1}) and sparged with air, were harvested and extracted as previously described (Whitney and Andrews, 2000). The protein fraction that precipitated between 8 and 20% (w/v) polyethylene glycol was collected for assay and decarbamylated as described above.

[^{14}C]-CABP carbamylation assay. Enzyme (30-40 µg ml^{-1}) was equilibrated with various concentrations of CO_2-free $MgCl_2$ and $NaHCO_3$ in N_2-flushed, septum-sealed vials at 25°C for 30 min. [^{14}C]-2'-carboxypentitol 1,5-bisphosphate (CPBP, an unresolved mixture of CABP and 2'-carboxyribitol 1,5-bisphosphate) in 30-fold excess of presumed enzyme active sites, was then added together with 20mM EDTA and allowed to bind for 5 min. In order to exchange loosely bound [^{14}C]-CABP, [^{12}C]-CPBP was added to the reaction mixture in 60-fold excess over [^{14}C]-CPBP. After a further 30 min, the mixture was transferred to ice. Protein-bound label was separated from unbound label by gel filtration through Sephadex G-50 and measured by scintillation counting.

$^{14}CO_2$ carbamylation assay. Tapered, septum-sealed vials containing 10 µl of $MgCl_2$ at varying concentrations were flushed with N_2 for 10 min before addition of 90 µl of an enzyme-$NaH^{14}CO_3$ pre-mix (16µM enzyme, 1.1 mM $NaH^{14}CO_3$ (130 Bq $nmol^{-1}$)). The mixture was left to equilibrate for 30 min at 25°C after which CPBP (20-fold excess over enzyme active sites) and $NaHCO_3$ (200-fold excess over $NaH^{14}CO_3$) were added, so that any carbamylation occurring subsequently was silent. After 30 min, the solution was transferred to ice and then gel filtered as described above.

Results and Discussion

Carbamylation measurements with [^{14}C]-CABP are unreliable unless unbound Mg^{2+} is sequestered when [^{14}C]-CABP is added. Initially, the [^{14}C]-CABP binding assay was carried out following the protocol of Hall et al (Hall, Pierce et al. 1981), which makes no attempt to sequester Mg^{2+} following addition of [^{14}C]-CABP. Data obtained in this manner with spinach Rubisco showed carbamylation rising steeply with increasing $MgCl_2$ concentration and reaching saturation by 2mM $MgCl_2$, even at low concentrations of $NaHCO_3$ (Figure 1a). The K_cK_{Mg} estimated from these results was a hundred times less than that reported previously for spinach Rubisco (1-2 x 10^5 µM^2, Laing and Christeller 1976).

High levels of carbamylation persisted even when CO_2 was rigorously excluded from all components of the activation solution (data not shown), suggesting that carbamylation and non-exchangeable [^{14}C]-CABP binding was occurring after addition of the labelled CABP, perhaps as a result of carbonate/bicarbonate unavoidably introduced with the CABP which is stored at alkaline pH to prevent lactonisation. Edmondson et al. (1990b) showed that CO_2 will promote carbamylation of uncarbamylated sites if added with or even 30 min after the CABP. Presumably this is because the complex between uncarbamylated sites and CABP is loose

enough to allow a concentration of uncomplexed, uncarbamylated sites sufficient to support a finite rate of carbamylation in the presence of CABP if both CO_2 and Mg^{2+} are also present. Such unwanted carbamylation occurring in the interval between addition of [^{14}C]-CABP and unlabelled CABP will spuriously inflate the observed measurement.

Carbamylation occurring after addition of [^{14}C]-CABP was effectively suppressed by adding EDTA (in 2-fold excess of the highest Mg^{2+} concentration) simultaneously with the [^{14}C]-CABP (a possibility suggested by the findings of Zhu and Jensen, 1990). Control experiments showed that this addition did not decarbamylate fully carbamylated enzyme (data not shown). When amended in this way, the assay reproduced the K_cK_{Mg} values observed in previous studies (Laing and Christeller 1976) (Figure 1b).

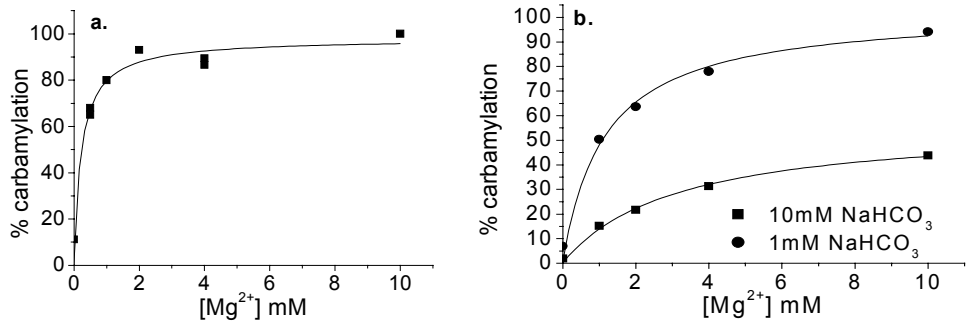

Figure 1: [^{14}C]-CABP carbamylation assays on spinach Rubisco with and without 20mM EDTA added with the [^{14}C]-CABP.

a. Assay conducted at 0.44 mM $NaHCO_3$ without EDTA addition. 30 min between addition of [^{14}C]-CABP and [^{12}C]-CABP. $K_cK_{Mg} = 1.8x10^3$
b. Assays conducted at 1 and 10 mM $NaHCO_3$ with EDTA added at the same time as [^{14}C]-CABP. 5 min between addition of [^{14}C]-CABP and [^{12}C]-CABP. $K_cK_{Mg} = 0.54x10^5$, $2.26x10^5$, respectively.

CABP binds non-exchangeably to uncarbamylated G. sulphuraria Rubisco. When subject to the [^{14}C]-CABP carbamylation assay, *G. sulphuraria* Rubisco responded differently to its higher plant counterpart. Firstly, activation by Mg^{2+} and CO_2 was not a pre-requisite for tight binding of CABP. Even when dialysed in the presence of EGTA, *G. sulphuraria* Rubisco bound [^{14}C]-CABP to apparently saturating levels without addition of either cofactor. The possibility that *G. sulphuraria* Rubisco bound the co-factors so tightly that they could not be removed by dialysis in the presence of EGTA was excluded by observations that the dialysed enzyme was inactive as a carboxylase unless Mg^{2+} was added. Some structural difference between the higher-plant and algal enzymes must cause the latter to bind CABP non-exchangeably regardless of carbamylation status. This finding might highlight a structural basis for *G. sulphuraria* Rubisco's differences in kinetic parameters in comparison to higher-plant Rubiscos (Whitney et al., 2001).

Does G. sulphuraria Rubisco have two different types of CABP-binding sites? Exchange with [^{12}C]-CABP, used in sub-saturating conditions to exchange loosely bound E-^{14}CABP and EC-^{14}CABP complexes, should have no effect when CO_2 and Mg^{2+} are saturating and all sites are in the ECM form. The exchange step is commonly omitted when CABP binding is used to estimate the total amount of Rubisco active sites in protein samples after full carbamylation. Carbamylation assays under saturating conditions (10mM Mg^{2+}, 50mM $NaHCO_3$) revealed a second profound difference between the higher-plant and red-algal Rubiscos. If the exchange with [^{12}C]-CABP was omitted, approximately twice as much [^{14}C]-CABP remained bound, whereas control measurements with spinach Rubisco under similar conditions showed that the exchange step made little difference (not shown). Indeed, approximately half of the [^{14}C]-

CABP bound by the *G. sulphuraria enzyme* at all cofactor concentrations, including zero, could be exchanged with [^{12}C]-CABP. Either only four of the *G. sulphuraria* Rubisco's eight active sites bind CABP tightly or each of the eight large subunits is capable of binding a second [^{14}C]-CABP molecule tightly enough to survive gel filtration but not to resist exchange with an excess of unlabelled CABP. In either scenario, CABP binding appears indifferent to carbamylation status. Experiments in which both the carbamylating CO_2 molecule and the CABP are labelled will be required to resolve this issue.

The carbamylation equilibrium of G. sulphuraria *Rubisco.* As a consequence of *G. sulphuraria* Rubisco's indiscriminate binding of CABP, estimations of ECM over a range of co-factor concentrations were achieved using a direct approach with labelled $NaH^{14}CO_3$ where further carbamylation after addition of unlabelled CABP was rendered silent by the simultaneous addition of a large excess of $NaH^{12}CO_3$. From this assay, the K_cK_{Mg} value for *G. sulphuraria* Rubisco was found to be slightly lower than for spinach (Figure 2).

Conclusion. These findings establish that the carbamylation properties of at least one red-algal Rubisco are compatible with the conditions in higher plant chloroplasts. Nevertheless,

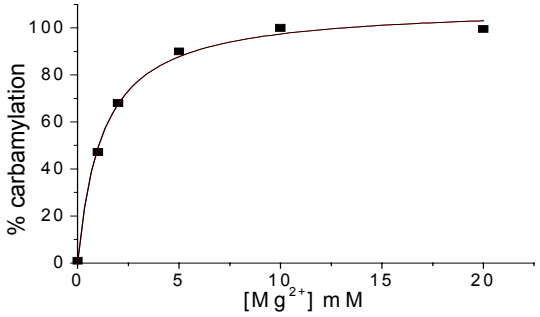

Figure 2: Carbamylation of *G. sulphuraria* Rubisco at 1mM $NaH^{14}CO_3$. Determined by $^{14}CO_2$ carbamylation assay (see Materials and Methods). $K_cK_{Mg} = 2.5x10^4$

there are profound differences in ligand binding properties between this enzyme and its well-studied counterpart from higher plants.

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