Regulation of Rubisco activase and its interaction with Rubisco

Archie R. Portis Jr\textsuperscript{1,2,*}, Cishan Li\textsuperscript{2,†}, Dafu Wang\textsuperscript{2} and Michael E. Salvucci\textsuperscript{3}

\textsuperscript{1} USDA-ARS, Photosynthesis Research Unit, Urbana, IL 61801, USA
\textsuperscript{2} University of Illinois, Department of Plant Biology, Urbana, IL 61801, USA
\textsuperscript{3} USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ 85239, USA

Received 31 July 2007; Revised 24 August 2007; Accepted 11 September 2007

Abstract

The large, \(\alpha\)-isoform of Rubisco activase confers redox regulation of the ATP/ADP response of the ATP hydrolysis and Rubisco activation activities of the multimeric activase holoenzyme complex. The \(\alpha\)-isoform has a C-terminal extension that contains the redox-sensitive cysteine residues and is characterized by a high content of acidic residues. Cross-linking and site-directed mutagenesis studies of the C-terminal extension that have provided new insights into the mechanism of redox regulation are reviewed. Also reviewed are new details about the interaction between activase and Rubisco and the likely mechanism of ‘activation’ that resulted from mutagenesis in a ‘Sensor 2’ domain of activase that AAA\textsuperscript{+} proteins often use for substrate recognition. Two activase residues in this domain were identified that are involved in Rubisco recognition. The results directly complement earlier studies that identified critical residues for activase recognition in the large subunit of Rubisco.

Key words: AAA\textsuperscript{+} protein, cross-linking, redox regulation, Rubisco.

Introduction

The activation state of Rubisco responds to light-mediated changes in stromal redox state and the phosphorylation potential determined by the stromal ATP/ADP ratio via regulation of the ATPase activity of Rubisco activase (Zhang and Portis, 1999; Zhang \textit{et al.}, 2002). The ATPase activity of activase is believed to power conformational changes in Rubisco that promote the release of inhibitory sugar phosphates from the catalytic site (reviewed in Portis, 2003). The ATPase activity of activase is very sensitive to the ATP/ADP ratio and this sensitivity in turn is modulated by thioredoxin-mediated dithiol/disulphide exchange between two cysteines in the C-terminal extension present in the \(\alpha\)-isoform of activase. However, the molecular details of how a redox change in the C-terminal extension alters the ATP/ADP sensitivity of ATP hydrolysis remain obscure, particularly in the absence of a three-dimensional structure for activase. For the work reviewed herein, site-directed mutagenesis and a cross-linking method were applied to test the hypothesis that redox-dependent conformational changes in the C-extension result in ionic interactions between negatively charged residues in the C-extension and positively charged residues present in or near the nucleotide-binding site.

The lack of a molecular structure for activase also continues to hinder progress in understanding how activase interacts with and promotes conformational changes in Rubisco. To compensate, the peculiar specificity of activase from tobacco (Wang \textit{et al.}, 1992), a plant in the family Solanaceae, and the knowledge that activase belongs to the AAA\textsuperscript{+} (ATPase associated with diverse cellular activities) protein family (Neuwald \textit{et al.}, 1999) were exploited. Early on, tobacco activase was found to be a rather poor activator of Rubisco from a wide range of plants outside the Solanaceae (Wang \textit{et al.}, 1992), while all activases and Rubiscos from families of non-solanaceous plants that have been examined thus far, including spinach, cotton, \textit{Arabidopsis}, and even \textit{Chlamydomonas}, are capable of interacting with each other. Subsequent...
research using site-directed mutagenesis of the large subunit of Rubisco in *Chlamydomonas* allowed the identification of two Rubisco residues that confer preference for tobacco versus spinach activase (Larsen et al., 1997; Ott et al., 2000). The knowledge that activase is an AAA protein, in which certain members use a specific C-terminal domain known as ‘Sensor 2’ for substrate recognition (McAlear et al., 1994; Smith et al., 1999), renewed and guided the interest in the identification of activase residues that might be involved, and that work is also reviewed.

### Mechanism of redox regulation of activase

Zhang et al. (2001) speculated that oxidation and formation of a disulphide bond between the cysteines in the C-extension of the α-isofom of activase cause a conformational change that allows docking near or into the nucleotide-binding site via one or more of the large number of negatively charged residues in the C-extension (Fig. 1A). Site-directed mutagenesis of the negatively charged residues and cross-linking/peptide identification were recently utilized to investigate this hypothesis further (Wang and Portis, 2006).

#### Negative residues in the C-extension are essential for altered ADP sensitivity

Alanine replacement was used to examine the importance of the negatively charged residues in the C-extension of *Arabidopsis* activase (Fig 1B). Among the six single alanine replacement mutants, only E390A, D394A, and D401A exhibited considerable changes in ATP hydrolysis activity in response to the ADP/ATP ratio (Fig. 1B). Two double mutants were then made and analysed to look for an enhanced effect. The ATP/ADP response of the E390A/D401A double mutant was substantially altered, exhibiting 4.6-fold higher ATP hydrolysis (and 6.6-fold higher Rubisco activation activity; not shown) at a typical ATP/ADP ratio (0.33) for the chloroplast stroma in the light (Fig. 1C). These changes in activity are about equal to those reported previously for redox treatment of the wild-type α-isofom (Zhang and Portis, 1999). Thus the results clearly indicate that negatively charged residues are an essential component of the redox regulation mechanism.

#### The C-extension can specifically alter ATP binding

Redox treatments of the α-isofom were found to alter its ATP affinity, suggesting that the nucleotide-binding site becomes more suitable for ATP binding after reduction (Zhang et al., 2001). Therefore, several different measurements were made of nucleotide binding for both ADP and ATP or an ATP analogue, ATP-γ-S, for the α- and β-isofoms and two α-isofom mutants. The measurements (Table 1) indicated that the binding affinities for ADP were all very similar, whereas reduction of the α-isofom increased the affinity for ATP and ATP-γ-S to values more similar to those of the small β-isofom. Most
Table 1. The effects of redox treatment and mutation on the nucleotide binding capacities of wild-type Arabidopsis Rubisco activase isoforms and mutants

The nucleotide binding of activase was estimated either using apparent dissociation constants ($K_d$) for ADP and ATP-$\gamma$-S by measuring fluorescence quenching of 1,8-ANS–activase complex and intrinsic fluorescence increase of activase in response to different nucleotide concentrations, respectively, or the ATP concentration for half-maximal ATP hydrolysis activity of activase. Data are taken from Wang and Portis (2006), and further details may be obtained from this reference.

<table>
<thead>
<tr>
<th>Rubisco activase</th>
<th>ANS fluorescence</th>
<th>Intrinsic fluorescence</th>
<th>ATP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$-ADP ((\mu M))</td>
<td>$K_d$-ATP-$\gamma$-S ((\mu M))</td>
<td>$K_m$ ((\mu M))</td>
</tr>
<tr>
<td>$\alpha$-Isoform-ox</td>
<td>3.2±0.2</td>
<td>15±0.6</td>
<td>11±0.4</td>
</tr>
<tr>
<td>$\alpha$-Isoform-red</td>
<td>2.9±0.1</td>
<td>9.3±0.4</td>
<td>6.8±0.3</td>
</tr>
<tr>
<td>$\alpha$-C411A</td>
<td>3.0±0.2</td>
<td>9.1±0.4</td>
<td>6.9±0.3</td>
</tr>
<tr>
<td>$\alpha$-E390A/D401A</td>
<td>3.1±0.2</td>
<td>9.4±0.3</td>
<td>7.5±0.3</td>
</tr>
<tr>
<td>$\beta$-Isoform</td>
<td>2.8±0.1</td>
<td>7.4±0.3</td>
<td>5.3±0.2</td>
</tr>
</tbody>
</table>

significantly, both the $\alpha$-isoform C411A mutant which cannot be redox regulated and the $\alpha$-isoform E390A/D401A mutant exhibited higher affinities for ATP and ATP-$\gamma$-S than the oxidized wild-type $\alpha$-isoform. These results clearly indicate that the binding of ATP, but not ADP, in the nucleotide-binding pocket is selectively impaired only when the cysteines in the C-extension can form a disulphide and a sufficient number of negatively charged residues are present that might allow specific interactions with other residues near the nucleotide-binding domain.

The C-extension of the oxidized $\alpha$-isoform is in close proximity to the nucleotide-binding pocket

To examine the proximity of the C-extension to other activase domains, a chemical modification approach was taken that utilized a sulphhydryl-reactive and photoactivated cross-linking reagent, PEAS, followed by biotin labelling, trypsin digestion, and affinity purification of the peptides. A new cysteine residue was inserted at position 402 (Fig. 1A) to allow chemical modification of the C-extension with PEAS, which has been successfully applied to examine other protein interactions (Pioszak et al., 2000; Cai et al., 2001; Ioth et al., 2001). The approach also relied on a previous observation that the endogenous cysteine residues, outside of the C-extension, are not very reactive (van de Loo and Salvucci, 1998; Salvucci, 2004). PEAS has an aryl azide group that becomes activated upon exposure to UV light and then reacts non-specifically by forming a nitrene, which undergoes ring expansion and reacts with nearby nucleophiles (Schuster and Platz, 1992). Reduction of the disulphide bond introduced by PEAS modification then releases the cross-linked products for proteolysis and peptide characterization by mass spectrometry.

Insertion of the cysteine had minimal effect on the ATP hydrolysis and Rubisco activation activities, and the redox midpoint potential of the mutant activase (C420INS) was similar to that of the wild type (Wang and Portis, 2006). Exposure of the PEAS-labelled C420INS mutant to UV light in the presence or absence of nucleotide resulted in formation of a high molecular weight band corresponding to a cross-linked multimer of activase and less intense bands corresponding to cross-linked dimers and trimers of activases (Fig. 2). Cross-linking between rather than within monomers also occurred under all conditions, as indicated by the marked reduction in intensities (72% for no-nucleotides, 69% for ADP, and 41% for ATP after 2 min exposure) of the monomer band. The cross-linking efficiency was very similar in the absence of nucleotides and in the presence of ADP, but lower when ATP was present. Thus ATP binding provides some protection against cross-linking.

As outlined (Fig. 3A), biotin derivatization and avidin–agarose affinity purification were used to isolate peptides containing only free -SH groups generated by reduction of either the endogenous disulphide bond in the oxidized activase or the disulphide bond introduced by PEAS modification. A representative mass spectrum of the biotin-derivatized peptides of C420INS is shown in Fig. 3B. Evidence that the C-extension in the oxidized $\alpha$-isoform is located near the nucleotide-binding site was provided by the presence of three modified peptides identified as VPLIL-GIWGKQGQK (98–112), NFTLPNIKVPLILGWGK (89–108), and MEK (241–243). The first two are part of a Walker A (P-loop) directly involved in nucleotide binding. The MEK (241–243) peptide is adjacent to an ‘Arg finger’ (R240) and contains a lysine (K243) residue previously shown to be essential for ATP hydrolysis, but not ATP binding, and both probably interact with the $\gamma$-phosphate of ATP (Salvucci and Klein, 1994; Li et al., 2006). Another peptide, IKDEIVTLVDQFPGQSIDFF-GALRAR (266–292), which contains part of the Sensor 2 motif, was observed in one of the three replications. An arginine residue (292) in this motif is critical for ATP hydrolysis and Rubisco activation, and also may project into the nucleotide-binding site (Li et al., 2006).

The extensive intersubunit cross-linking observed (Fig. 2) is consistent with the observation that the nucleotide-binding pocket in many AAA$^+$ proteins usually consists of residues from adjacent subunits arranged in oligomeric assemblies (Neuwald et al., 1999; Ogura and Wilkinson, 2001). Also partial intersubunit cross-linking was observed when the C420INS mutant and wild-type redox-insensitive $\beta$-isoform were mixed (Wang and Portis, 2006). This suggests that the interactions between the two isoforms include the C-extension in its oxidized state. Such an interaction may explain how the $\alpha$-isoform
can regulate both isoforms in vitro and in vivo (Zhang and Portis, 1999; Zhang et al., 2002).

**A model for the interaction of the C-extension with the nucleotide-binding pocket**

A hypothetical model for how the C-extension may interact with the nucleotide-binding pocket in activase is presented in Fig. 4. Three peptides (N89-K108, V98-K112, and M241-K243; green) that cross-linked with the C-extension contribute residues to form the nucleotide-binding pocket, which is probably formed from amino acids in adjacent subunits (labelled Subunit 1 and 2). In the smaller, β-isoform or the reduced α-isoform where the C-extension is either absent or moves away from the nucleotide-binding pocket, the side chains of K108 and K112 (Subunit 1) and K243 and R240 (‘Arg finger’) (Subunit 2) point to the nucleotide-binding pocket, favouring occupation by ATP (red). In the oxidized α-isoform with two cysteines forming a disulphide (C392/C411, yellow), the C-extension docks into or adjacent to the nucleotide-binding pocket and interferes with the proper interactions with ATP by electrostatic interactions between its negative residues (E390, D394, and D401) and the positive residues mentioned above.

It is interesting that structural studies of glyceraldehyde-3-phosphate dehydrogenase (Fermani et al., 2007) revealed that its regulation depends on the docking of a disulphide-structured, negatively charged C-extension in the nucleotide-binding region that alters the specificity for NADPH versus NADP. A similar docking mechanism for the C-terminal extension of NADP-malate dehydrogenase was proposed earlier (Carr et al., 1999) from structural studies.

**Identification of activase residues involved in Rubisco preference**

Residues located at position 89 and 94 (spinach numbering) were previously identified as critical for the unique species specificity of the tobacco (Solanaceae) Rubisco–activase interaction (Larson et al., 1997; Ott et al., 2000). This approach utilized the capability to make site-directed mutations in the large subunit of *Chlamydomonas* Rubisco and comparisons of its ability to be activated by spinach and tobacco activase. Two separate point mutations, P89R and D94K, greatly improved its activation by tobacco activase, while severely impairing its activation by spinach activase. Attempts were also made to identify domains in activase by constructing chimeric spinach–tobacco activases and comparing their abilities to activate spinach and tobacco Rubisco (Esau et al., 1998). This
study indicated that the major determinants of Rubisco preference were located in a domain C-terminal to the nucleotide-binding domain. Many amino acid differences exist in this area and, without more information about the three-dimensional structure of activase, studies with additional chimeras or site-directed mutants were not pursued. However, the identification of activase as a member of the AAA+ protein family (Neuwald et al., 1999) and the ability to model the conserved AAA+ sequence–structure motifs (Bystroff HMMSTR, a hidden Markov model based on the I-sites Library of was superimposed. The structure of the C-extension was predicted using heat shock protein Hslu (1G4l) as the template (see Portis, 2003) and for clarity, some modelled regions surrounding the core nucleotide-binding pocket in activase. Details are discussed in the text. 

Fig. 4. A model for how the C-extension may interact with the nucleotide-binding pocket in activase. Details are discussed in the text. 

For clarity, some modelled regions surrounding the core β-sheets are omitted. The activase domains were modelled using the structure of the heat shock protein Hslu (1G4l) as the template (see Portis, 2003) and ATP from the structure of the N-ethylmaleimide-sensitive factor (INSF) was superimposed. The structure of the C-extension was predicted using HMMSTR, a hidden Markov model based on the I-sites Library of sequence–structure motifs (Bystroff et al., 2000).

Table 2. Spinach and tobacco Rubisco activation by wild-type tobacco and spinach activases and tobacco (T) and spinach (S) activase mutants

Rubisco activation, which was measured with 5 μg ml⁻¹ spinach or tobacco Rubisco and 80 μg ml⁻¹ of the indicated activase, was measured by the fraction of Rubisco sites activated after 6 min. Data are taken from Li et al. (2005), and further details may be obtained from this reference.

<table>
<thead>
<tr>
<th>Activase</th>
<th>Spinach Rubisco (fraction of sites activated)</th>
<th>Tobacco Rubisco (fraction of sites activated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>0.16±0.06</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td>T_D311K</td>
<td>0.40±0.03</td>
<td>0.48±0.04</td>
</tr>
<tr>
<td>T_L314V</td>
<td>0.05±0.04</td>
<td>0.53±0.04</td>
</tr>
<tr>
<td>T_D311K/L314V</td>
<td>0.39±0.02</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>S_K311D</td>
<td>0.08±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>Spinach</td>
<td>0.71±0.05</td>
<td>0.13±0.05</td>
</tr>
</tbody>
</table>

Table 3. Cotton and tobacco Rubisco activation by activases from tobacco, cotton, and cotton mutants (C)

Rubisco activation, which was measured with 500 μg ml⁻¹ cotton or tobacco Rubisco and 500 μg ml⁻¹ of the indicated activase, was measured by the fraction of Rubisco sites activated after 6 min. Data are taken from Li et al. (2005), and further details may be obtained from this reference.

<table>
<thead>
<tr>
<th>Activase</th>
<th>Cotton Rubisco (fraction of sites activated)</th>
<th>Tobacco Rubisco (fraction of sites activated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>0.67±0.02</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>C_K311D</td>
<td>0.36±0.01</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>C_V314L</td>
<td>0.57±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>C_K311D/V314L</td>
<td>0.12±0.01</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>Tobacco</td>
<td>0.11±0.04</td>
<td>0.65±0.02</td>
</tr>
</tbody>
</table>
examined. Wild-type cotton activated cotton Rubisco better than tobacco Rubisco, and vice versa. The cotton K311D (C^K311D) and to a lesser extent the V314L (C^V314L) mutations decreased the ability to activate cotton Rubisco and enhanced the ability to activate tobacco Rubisco. Complementary to the results with the tobacco double mutant, the cotton double mutant, C^K311D/V314L exhibited a better ability to activate tobacco Rubisco as compared with C^K311D, and it exhibited a reduced ability to activate cotton Rubisco.

The identification of residues 311 and 314 as important in determining the specificity of tobacco activase for Rubisco raised the possibility that the mutations in the large subunit of Chlamydomonas Rubisco that altered its activase preference could be complemented by the tobacco activase mutations. As shown in Fig. 5 and similarly to previous reports (Larson et al., 1997; Ott et al., 2000), tobacco activase activated Chlamydomonas P89R and D94K Rubisco, but not the wild type. The mutant tobacco activase T^D311K activated wild-type Rubisco and P89R, but the activation of D94K Rubisco was reduced. In contrast, the tobacco activase mutant T^L311V activated D94K Rubisco quite well, but it was a poor activator of P89R Rubisco and the wild type. Finally, and unlike wild-type tobacco activase, the double mutant T^D311K/L314V activated wild-type Chlamydomonas Rubisco much better than it activated either the P89R or D94K Rubisco mutants, thus exhibiting a preference similar to spinach activase.

A simple alignment and comparison of the amino acid sequences of tobacco (Solanaceae) and non-solanaceous activase and Rubisco provides an interesting view of the specificity results (Fig. 6). The charge switch at position 94 in Rubisco and 311 in activase suggests that these residues may directly interact. There may also be a steric interaction between the residues at position 89 in Rubisco and 314 in activase given that the specific residues at this position can also alter or modify the specificity preference in some contexts. Although the other residues are highly conserved on both proteins, the negatively charged E93 in Rubisco is potentially complementary to the positively charged K (or in some cases R) at position 312 in activase. This pair could be interesting for future mutagenesis experiments. The specificity-determining interactions identified in this work provide some guidance for the current interest in genetically engineering Rubisco and the activase to increase photosynthesis. To maintain a high efficiency in the interaction and the activation process, both proteins may have to be modified in a co-ordinated manner.

---

**Fig. 5.** Carbamylation rate of Chlamydomonas Rubisco from wild type, mutant P89R, and mutant D94K with increasing concentrations of wild-type tobacco Rubisco activase (A), and tobacco Rubisco activase mutants D311K (B), L314V (C), and the double mutant D311K/L314V (D). Data are taken from Li et al. (2005), and further details may be obtained from this reference [figure modified from Li et al. (2005) and is reproduced by kind permission of the American Society for Biochemistry and Molecular Biology Inc.].
A model for Rubisco activation by activase

It was noted previously that changes in the position of the N-terminus of Rubisco, including the interaction/specificity region 89–94, that occur as the molecule assumes the closed and open conformations (Duff et al., 2000) provide an insight into how activase may exert its effect on the sugar phosphate-binding site (Portis, 2003). Once bound to Rubisco through electrostatic and other forces that clearly include the region 89–94 on Rubisco and the region 311–314 on activase, ATP hydrolysis probably promotes movement of the C-terminal Sensor 2 domain (including region 311–314) of activase, as occurs for other AAA\(^*\) ATPases. This domain contains a critical arginine residue which is required for the ATPase activity of activase (Li et al., 2006) like many other AAA\(^*\) proteins. In these proteins, this arginine interacts with the bound nucleotide (Ogura and Wilkinson, 2001; Ogura et al., 2004). If this is also true for activase, it provides a direct way to couple ATP hydrolysis with the movement of the Sensor 2 region on activase and thus the movement of the N-terminal domain of Rubisco via the interactions established at positions 89–94 and probably elsewhere. A key feature of the movement of the Rubisco N-terminal domain could be the break in the interactions between Glu60 in the N-terminal domain of Rubisco, Lys334 in the C-terminal Sensor 2 domain on activase and thus the movement of the N-terminal domain could be the break in the interactions between the two encoded proteins.

References


Acknowledgements

We thank Dr RJ Spreitzer (University of Nebraska) for the collaborative research that provided the Chlamydomonas strains used in the work reviewed here. Research in our laboratories is supported by the USDA and a grant from the United States Department of Energy (97ER20268).


