Potential for interactions between the carboxy- and amino-termini of Rubisco activase subunits[1]

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Abstract The subunit interactions of Rubisco activase were investigated using mutants containing an introduced Cys near the N- and/or C-terminus. Chemical cross-linking of the C-terminal and double insertion mutant produced subunit dimers and dimers plus high ordered oligomers, respectively. Fluorescence measurements with *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine showed that the environment around the introduced Cys near the C-terminus becomes more hydrophilic upon nucleotide binding. The Cys insertion mutants catalyzed Rubisco activation and ATP hydrolysis even when the subunits of the C-terminal or double insertion mutants were completely cross-linked. The results indicate that the termini of adjacent activase subunits are in close proximity and can be modified and even joined without affecting enzyme function.

Key words: AAA$^+$ protein; ATPase; Carbon metabolism; Cross-linking; Photosynthesis

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from higher plants and eukaryotic algae is susceptible to a type of mechanism-based inactivation [1–3]. During the normal course of catalysis, the binding of the sugar-phosphate substrate, ribulose 1,5-bisphosphate (RuBP), to carbamylated sites containing Mg^{2+} triggers conformational changes that shield the active site from solvent, providing a closed environment for the fixation of CO_2 or O_2 [4,5]. On occasion, Rubisco sites that are catalytically inactive can assume this 'closed' conformation, for instance when uncarbamylated sites bind RuBP or when the enediol reaction intermediate is misprotonated at the site producing an inhibitory substrate analog [6]. Reopening of the closed conformation is very slow in the absence of catalysis, requiring a specific molecular chaperone called Rubisco activase to convert closed, catalytically inactive sites to an open conformation [1–3]. This action, which is dependent on the ATP/ADP ratio and redox status of the chloroplast [3], ultimately determines the proportion of Rubisco sites available for catalysis. Consequently, the controlled switching of Rubisco from the closed to the open conformation by activase serves an important regulatory function in photosynthesis, adjusting the rate of CO_2 fixation to the rate of electron transport activity.

Activase is an AAA$^+$ protein [7] with a single ATP binding site per subunit. Comparison of the primary structure of activase with other AAA$^+$ proteins has shown that the central portion of the linear activase molecule constitutes the AAA$^+$ module with characteristic features for nucleotide binding and hydrolysis. In fact, this region of the protein has been modeled using the 3-D structure of the Hsul protease as a template [3]. Various lines of evidence indicate that residues involved in the interaction with Rubisco are located outside the region of homology with other AAA$^+$ proteins at both the C- and the N-terminus of activase. Thus far, efforts to derive a 3-D structure of Rubisco activase using crystallography have not been forthcoming, frustrated in all likelihood by the highly polydispersed nature of the protein [8,9].

Like other AAA$^+$ proteins the active form of activase is a higher ordered oligomer with as many as 16 subunits in its most active form [3,9,10]. Since these subunits interact cooperatively for both ATP hydrolysis and Rubisco activation [8,9,11], the nature of these interactions is of interest for understanding activase function. Here we describe the results of directed mutagenesis, cross-linking and fluorescence experiments that examined the proximity of the N- and C-terminal regions of the protein and investigated their roles in activase function.

2. Materials and methods

2.1. Materials

N,*N*′-Dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamine (I-NBD) was purchased from Molecular Probes (Eugene, OR, USA). Bis-maleimidoethane (BMOE), 1,4-bis-maleimidobutane (BMB), 1,8-bis-maleimidotriethyleneglycol (BM[PEO]$_3$), sulfosuccinimidyl[4-iodoacetyl]aminobenzoate (sulfo-SIAB) and *N*-[κ-maleimidoundecanoyloxy]-sulfosuccinimide ester (sulfo-KMUS) were obtained from Pierce Biotechnology (Rockford, IL, USA). The reagents were dissolved in either water (BM[PEO]$_3$, sulfo-SIAB, sulfo-KMUS) or dimethylformamide (BMOE, BMB, I-NBD) at a concentration of 5 mM.

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Abbreviations: BMOE, bis-maleimidoethane; DTT, dithiothreitol; I-NBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; NBD, nitrobenz-2-oxa-1,3-diazole; RuBP, ribulose 1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase

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2.2. Directed mutagenesis

Nucleotides encoding Cys and Ala were inserted near the 5′- and 3′-ends of the coding region of a cDNA clone for the β form of cotton activase [12] by polymerase chain reaction (PCR) using the mutagenic primers 5′-CTACCATGGCGTGTGCGAAAGAGATAGACGAAG-AC and 5′-GTGAATTCTCATTTAGAACGCACAAGCTCCTCTC-TTGATAGCA (Table 1). Insertion mutants containing a Trp or Leu at position 379 were produced by PCR using the mutagenic primers 5′-CATTTAGAAAGCCCATCCTCTCTTGATAGC and 5′-CATT-TAGAAAGCCAATCCTCTCTTGATAGC, respectively. The PCR products were ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and the construct was used to transformed *Escherichia coli* TOP1-F′ cells. Activase cDNAs were excised by digestion with *Nco*I and *Eco*RI and subcloned into the pET 23d vector (Novagen, Madison, WI, USA) for protein expression in *E. coli* BL21(DE3)pLysS cells [13]. Mutations were confirmed by sequencing both strands of the cDNA clones.

2.3. Protein purification

Recombinant activase was purified from *E. coli* cells as described previously [12]. For peptide analysis, desalted proteins were digested overnight with cyanogen bromide [14]. The resulting peptides were separated by reverse phase high performance liquid chromatography [15] on a 4.6×250 mm 5 μ Jupiter C18 column (Phenomenex, Torrance, CA, USA) to confirm the presence of the desired insertions and to assess the extent of chemical modification with I-NBD. Rubisco was isolated from cotton leaves as described previously [16].

2.4. Cross-linking and chemical modification

Purified, recombinant activase was precipitated by the addition of an equal volume of saturated ammonium sulfate, adjusted to pH 7 with ammonium hydroxide. Precipitated protein was collected by centrifugation, suspended in 0.2 ml of 50 mM potassium phosphate buffer, pH 7.6, and desalted into this buffer by centrifugal gel filtration [17]. Following desalting, the protein (47.6 μM) was incubated at 10°C for 60 min with 5 mM ATP and a two-fold molar excess of reagent. Control reactions were supplemented with 10 mM dithiothreitol (DTT) prior to the addition of reagent. Reactions were terminated by the addition of DTT to 10 mM and the modified protein was precipitated by addition of ammonium sulfate as described above. Precipitated protein was collected by centrifugation, suspended in 0.2 ml of 50 mM HEPES-KOH, pH 7.2, and 10 mM $MgCl_2$ and separated from unincorporated reagent by centrifugal gel filtration. The desalted protein was stored at −80°C. Incorporation of nitrobenz-2-oxa-1,3-diazole (NBD) into activase was determined by absorption at 500 nm in 6 M guanidine-HCl using an extinction coefficient of 20 711 M^{-1}.

2.5. Analysis of cross-linked and chemically modified activase

The rates of ATP hydrolysis by activase were determined spectrophotometrically [16]. Activation of inactive Rubisco complexed with RuBP was determined after 0.5 and 5 min as described previously [16] using 0.5 mg/ml (7.14 μM protomer) Rubisco and 0.1 mg/ml (2.4 μM monomer) activase. Assays were conducted in triplicate and the results presented are the means ± S.E.M. Cross-linked protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels [8]. Fluorescence of NBD-modified activase was determined as described previously [13]. The concentration of activase protein was determined by a dye binding assay [18].

3. Results

3.1. Mutation of activase by insertion of Cys

Cys residues were inserted near the N- and/or C-termini of activase, regions of the protein thought to be involved in the interaction with Rubisco. Since the second and penultimate residues in the recombinant protein are both Ala, an Ala was inserted in the position adjacent to the Cys as a spacer. The nomenclature of the single and double Ala-Cys insertion mutants is presented in Table 1 along with their N- and C-terminal sequences.

3.2. Cross-linking of mutant activases

Initial experiments with the Ala-Cys insertion mutants of activase focused on attempts to obtain specific cross-links between activase and Rubisco using the homobifunctional sulfhydryl-reactive reagent, $BM[PEO]_3$, and the heterobifunctional (i.e. sulfhydryl- and amine-reactive) reagents, sulfo-SIAB and sulfo-KMUS. No specific activase–Rubisco cross-links were apparent on SDS–PAGE gels after incubation of wild type activase or either of the single insertion mutants with Rubisco in the presence of the cross-linking reagents (data not shown). Cross-links were also not formed between the insertion mutants and the α form of wild type cotton activase even though the α form of activase contains two Cys residues in its C-terminal extension [12] and $BM[PEO]_3$ was used for cross-linking (data not shown),

Analysis of control samples containing only the β form of activase showed that subunits of the C-terminal insertion mutant, $+A^{379}C^{380}$, were extensively cross-linked by $BM[PEO]_3$, a homobifunctional, sulfhydryl-directed reagent with a 14.7 Å spacer arm (data not shown). Additional experiments with BMB and BMOE, sulfhydryl-directed cross-linking reagents with 10.9 and 8 Å spacer arms, respectively, cross-linked the subunits of the $+A^{379}C^{380}$ mutant with the same efficiency as $BM[PEO]_3$ (data not shown). Consequently, cross-linking experiments were routinely performed with BMOE, the reagent with the shortest spacer arm.

No cross-linking was observed when wild type activase was incubated with BMOE (Fig. 1). In contrast, nearly 100% of the C-terminal mutant protein, $+A^{379}C^{380}$, was cross-linked by BMOE, producing a slower electrophoretic species that migrated on SDS–PAGE with an apparent molecular mass equivalent to an activase dimer. The N-terminal insertion mutant, $+A^2C^3$, was also cross-linked with BMOE, but to a very limited extent compared with the $+A^{379}C^{380}$ mutant. The use of $BM[PEO]_3$, a cross-linking reagent that is functionally similar to BMOE, but with a longer spacer arm, did not increase the cross-linking efficiency of the $+A^2C^3$ mutant (data not shown). The $+A^2C^3/A^{379}C^{380}$ double mutant with an Ala-Cys insertion near both ends of the protein was cross-linked with high efficiency, producing electrophoretic

Table 1
Nomenclature and N- and C-terminal sequences of wild type and mutant activases

Mutation	Nomenclature	Amino acid sequence	
		N-terminus	C-terminus
None	wild type	MAKEIDED…	…DDAIKRGAF
N-terminal Ala-Cys insertion	$+A^2C^3$	M<u>AC</u>AKEIDED…	…DDAIKRGAF
C-terminal Ala-Cys insertion	$+A^{379}C^{380}$	MAKEIDED…	…DDAIKRG<u>AC</u>AF
N- and C-terminal Ala-Cys insertion	$+A^2C^3/A^{379}C^{380}$	M<u>AC</u>AKEIDED…	…DDAIKRG<u>AC</u>AF

The inserted residues are underlined.

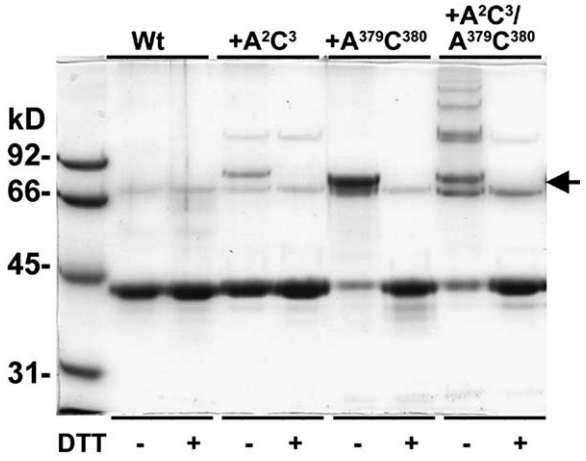

Fig. 1. Chemical cross-linking of the Cys insertion mutants of cotton activase. Wild type (Wt) and mutant activase protein was incubated with BMOE alone (−) or in the presence (+) of DTT. Reactions were terminated by addition of DTT and SDS and then heated to 95°C for 2 min. Polypeptides were separated by SDS–PAGE and visualized by staining with Coomassie brilliant blue. The M_r of standard proteins electrophoresed in lane 1 are indicated by the numbers to the left of the gel. The arrow indicates the position of the 84 kDa activase dimer. The 70 kDa polypeptide visible in all samples was identified by protein sequencing as *E. coli* DnaK, a molecular chaperone that co-purifies with recombinant activase.

species equivalent to subunit dimers, as well as higher ordered oligomers up to at least hexamers. In all cases, the presence of DTT in the reaction prevented cross-linking by BMOE.

3.3. Chemical modification of the activase mutants

To investigate the molecular environment of the C- and N-termini of activase, the Cys insertion mutants were chemically modified with I-NBD, an environmentally sensitive fluorophore. The amount of NBD associated with wild type activase was 0.18 mol/mol compared with 0.35, 0.85 and 1.09 mol/mol for the $+A^2C^3$, $+A^{379}C^{380}$, and $+A^2C^3/A^{379}C^{380}$ mutants, respectively, based on absorbance at 500 nm. Analysis of the peptides after digestion with cyanogen bromide revealed that the C-terminal peptide of the $+A^{379}C^{380}$ and $+A^2C^3/A^{379}C^{380}$ mutants, both of which contain a Cys insertion at position 380, was almost completely modified by NBD (data not shown). In contrast, the amount of NBD incorporated into the inserted Cys at the N-terminus of $+A^2C^3$ and $+A^2C^3/+A^{379}C^{380}$ was less than 10% on a mol/mol basis. Peptide analysis revealed that none of the NBD associated with wild type protein was covalently bound to the protein (data not shown).

The C-terminal mutant, $+A^{379}C^{380}$, and the double mutant, $+A^2C^3/A^{379}C^{380}$, both possessed high 430 nm fluorescence after chemical modification with I-NBD. Fluorescence from covalently bound NBD decreased markedly upon addition of ATP (Fig. 2) or ADP (data not shown) to a similar level for the two mutants. The fluorescence of NBD-modified $+A^{379}C^{380}$ was low and unaffected by nucleotide when the protein was denatured with guanidine-HCl. The fluorescence of the nucleotide-free $+A^{379}C^{380}$ mutant also decreased upon thermal denaturation of the protein (inset).

3.4. Effect of Ala-Cys insertions, cross-linking and chemical modification on activase activity

All three of the Cys insertion mutants were catalytically active, catalyzing ATP hydrolysis and promoting activation of Rubisco (Tables 2 and 3). The rates of ATP hydrolysis and Rubisco activation by the unmodified mutant proteins were lower than the wild type. However, the $+A^{379}C^{380}$ mutant and, to a more limited extent, the $+A^2C^3/A^{379}C^{380}$ mutant catalyzed significantly higher rates of ATP hydrolysis and Rubisco activation after chemical modification with I-NBD or cross-linking with BMOE. As a result, the rates of ATP hydrolysis by the extensively cross-linked $+A^{379}C^{380}$ mutant enzyme was even higher than the wild type.

3.5. Trp and Leu insertions at the C-terminus of activase

Because of the stimulation of activase activities upon chemical modification of the $+A^{379}C^{380}$ mutant with NBD, Trp and Leu residues were inserted at position 379 (i.e. $+L^{379}$ and $+W^{379}$) to examine the effect on activase function. The activities (ATPase and Rubisco activation) of the mutated proteins were indistinguishable from wild type (data not shown).

4. Discussion

The ATPase domain of activase, which encompasses nearly half of the 379 amino acids in the mature, non-recombinant β form of cotton activase, is highly homologous to similar domains on other AAA^+ proteins. This domain is located almost squarely in the central portion of the linear activase molecule, flanked on each end by regions that show little similarity to other AAA^+ proteins. Truncation and directed mutations have shown that the N-terminus, particularly a conserved W at position 16, is involved in the activation of Rubisco [11,19]. Removal of 19 amino acids from the C-terminus of the β form of spinach activase enhanced the ability

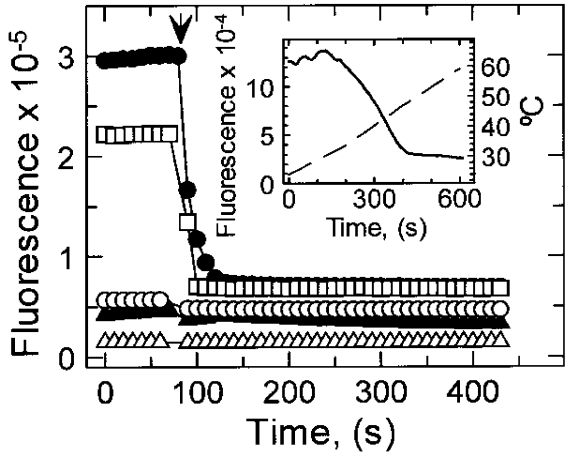

Fig. 2. Effect of nucleotide on the NBD fluorescence of the Cys insertion mutants of cotton activase. Fluorescence at 430 nm was determined over time in 400 μl reactions containing 50 μg wild type activase (△) or the Cys insertion mutants, $+A^2C^3$ (▲), $+A^{379}C^{380}$ (●) and $+A^2C^3/A^{379}C^{380}$ (□) after modification of the proteins with I-NBD. Fluorescence of the I-NBD-modified $+A^{379}C^{380}$ mutant was also measured in the presence of guanidine-HCl (○). Nucleotide in the form of 1 mM ATP was added at the time indicated by the arrow. Inset: Effect of thermal denaturation on the fluorescence of 20 μg of I-NBD-modified $+A^{379}C^{380}$ mutant. NBD fluorescence (solid line) was measured in the absence of nucleotide during a time course of gradually increasing temperature (dashed line).

Table 2
Effect of mutation, chemical modification and cross-linking on the ATPase activities of the Ala-Cys insertion mutants of activase

Activase	ATPase activity (U/mg protein)		
	No modification	NBD-modified[a]	BMOE-cross-linked[a]
Wild type	0.36 ± 0.02		
$+A^{379}C^{380}$	0.29 ± 0.01	0.54 ± 0.03	0.63 ± 0.04
$+A^{2}C^{3}$	0.30 ± 0.03		
$+A^{2}C^{3}/A^{379}C^{380}$	0.25 ± 0.01	0.38 ± 0.02	0.55 ± 0.02

[a]Wild type activase and the $+A^{2}C^{3}$ mutant were not modified with NBD or BMOE.

of activase to activate Rubisco but had little effect on its ATPase activity [19]. Construction of chimeric activases showed that the major determinants of selectivity for a particular Rubisco target reside primarily in the C-terminal region of activase [20]. These results are consistent with the general model of AAA^{+} action where cellular function, in this case the interaction with Rubisco, is determined by domains outside the conserved ATPase module [7].

The β form of cotton activase contains four Cys residues that are generally conserved among the β form activases from most plant species [12]. Previous studies with the tobacco enzyme showed that the wild type enzyme was not modified by I-NBD, an indication that all four Cys residues are buried within the molecule and inaccessible to solvent [11]. A similar conclusion can be reached from the lack of covalent incorporation of NBD into wild type cotton activase (Fig. 2). In contrast, a Cys inserted three residues from the C-terminus of activase was thoroughly modified by I-NBD, indicating that this residue was readily accessible to solvent. The decrease in NBD fluorescence observed upon nucleotide binding indicates that the environment of this Cys becomes considerably more hydrophilic upon nucleotide binding.

The presence of an unmodified Cys three residues from the C-terminus of activase had only a minor effect on activase activity. Similarly, insertion of a Trp or Leu at this position did not affect ATP hydrolysis or alter the interaction of activase with Rubisco. Previously, a more drastic modification, the deletion of 20 amino acids, caused a marked increase in the ability of activase to promote Rubisco activation [19]. In the present study, chemical modification of the C-terminal insertion mutant, $+A^{379}C^{380}$, with NBD almost doubled both ATP hydrolysis and Rubisco activation activity, improving the rate even over the wild type. Taken together, these results suggest that residues of the C-terminus of activase, even those near the very end of the protein, can exert a subtle influence over activase activity.

Insertion of a Cys residue near the C-terminus of activase facilitated analysis of the proximity of this region on adjacent subunits using chemical cross-linking. While no apparent cross-links were formed with Rubisco, nearly complete intersubunit cross-linking was observed with activase. This finding makes it possible to conclude that the C-termini of adjacent activase subunits lie within a proximity of 8 Å or less. Interestingly, nearly complete cross-linking of activase at the C-terminus or at both the C- and N-termini (see below) actually increased ATPase activity and the ability of activase to promote activation of Rubisco. These results indicate that the activase mechanism does not require an exchange of monomeric subunits during cycles of ATP hydrolysis as has been proposed [10].

The low efficiency at which the N-terminal Cys insertion was modified by I-NBD compared with the C-terminal insertion indicates that a Cys residue in the third position of activase is either much less accessible to solvent than a Cys inserted near the C-terminus or much less reactive. The $+A^{2}C^{3}$ mutant protein was cross-linked with BMOE at very low frequency, whereas the double insertion mutant was extensively cross-linked producing dimers, as well as significant amounts of higher ordered oligomers. Conceivably, modification of the C-terminal Cys in the double mutant by the cross-linking reagent enhanced the accessibility of the N-terminal Cys insertion. This explanation could account for why the double insertion mutant was extensively cross-linked, even though the efficiency of I-NBD modification of Cys3 in the $+A^{2}C^{3}$ and $+A^{2}C^{3}/A^{379}C^{380}$ mutants was low.

The conditions used for here for cross-linking, i.e. high protein concentration and the presence of ATP, promote the most highly associated form of activase [8,9]. Gel filtration chromatography of the $+A^{379}C^{380}$ and $+A^{2}C^{3}/A^{379}C^{380}$ mutants in the presence of ATP showed that greater than 80% of the activase subunits were associated as at least tetramers and that cross-linking was accompanied by both a shift in the

Table 3
Effect of mutation, chemical modification and cross-linking on Rubisco activation by the Ala-Cys insertion mutants of activase

Activase	Rubisco activation[a] (fraction of sites made catalytically competent/min)		
	No modification	NBD-modified[b]	BMOE-cross-linked[b]
Wild type	0.13 ± 0.02 (0.49 ± 0.04)[c]		
$+A^{379}C^{380}$	0.09 ± 0.01 (0.43 ± 0.02)	0.17 ± 0.01 (0.64 ± 0.01)	0.16 ± 0.01 (0.65 ± 0.04)
$+A^{2}C^{3}$	0.07 ± 0.02 (0.29 ± 0.01)		
$+A^{2}C^{3}/A^{379}C^{380}$	0.08 ± 0.01 (0.37 ± 0.01)	0.12 ± 0.01 (0.50 ± 0.05)	0.10 ± 0.01 (0.52 ± 0.03)

[a]Initial rate of Rubisco activation. The rate of spontaneous activation by the no activase controls was 0.02 sites/min.
[b]Wild type activase and the $+A^{2}C^{3}$ mutant were not modified with NBD or BMOE.
[c]The values in parentheses indicate the final extent of activation, i.e. the fraction of sites made catalytically competent after 5 min. The extent of spontaneous activation by the no activase controls was 0.05 sites/5 min.

average distribution of activase towards even higher ordered oligomers (data not shown) and an improvement in activity. These results, together with the almost complete cross-linking of the $+A^{379}C^{380}$ and $+A^{2}C^{3}/A^{379}C^{380}$ mutant subunits, indicate that cross-linking of activase subunits probably occurred between subunits that were associated in a functional oligomer rather than between unassembled activase monomers. The cross-linking patterns of the $+A^{379}C^{380}$ and $+A^{2}C^{3}/A^{379}C^{380}$ mutants suggest an arrangement of activase subunits where (1) the C-termini of an adjacent pair of activase subunits are in close (i.e. <8 Å) proximity and (2) the N-terminus of a subunit outside this pair is within this distance of either the C- or the N-terminus of this pair. Such an arrangement of activase subunits is consistent with the typical assembly of AAA^{+} monomers in ring structures composed of multiple subunits [21].

5. Conclusions

The results presented here showed that activase is quite amenable to modification, including extensive intersubunit cross-linking, without loss of activity. The insertions introduced into the protein were outside the ATPase domain, involving regions that are thought to be important for the interaction with Rubisco. The results showed that it is possible to manipulate activase structure considerably without affecting the enzyme's ability to catalyze ATP hydrolysis or promote conformational changes in Rubisco. Since activase is an early target of heat stress [16], changes in activase structure that improve its thermal stability could provide a means of improving the thermotolerance of photosynthesis. If thermal denaturation of activase is preceded by subunit dissociation [22], then increasing the stability of the most highly associated form of activase represents a possible strategy for improving the thermal stability of activase.

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