Involvement of Two Aspartate Residues of Rubisco Activase in Coordination of the ATP γ-Phosphate and Subunit Cooperativity

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ABSTRACT: Aspartate residues are involved in coordination of the nucleotide−metal of several nucleotide triphosphatases. To examine interactions between Rubisco activase and ATP, site-directed mutations were made at two species-invariant aspartate residues, D174 and D231. In the absence of the magnesium cofactor, the mutant proteins D231R, D174Q, and D174A, but not D174E, bound ATP with higher affinity than did wild-type. In the presence of Mg^{2+}, the affinity for ATP of D231R was further increased, but was reduced with mutations at D174. Although all mutants bound ATP, only D174E aggregated in response to ATP/Mg^{2+} and retained partial ATPase and Rubisco activation activities. In mixing experiments, the catalytically competent D174E stimulated wild-type ATPase activity, whereas the mutants lacking ATPase activity were inhibitory to wild-type enzyme and prevented aggregation. These results are consistent with a mechanism for activase that involves ATP-binding, subunit aggregation and ATP hydrolysis as sequential steps in the catalytic mechanism. The results also indicated that precise coordination of the γ-phosphate is required for aggregation and depends on D174 and D231. To account for the pronounced cooperativity of Rubisco activase subunits, we suggest that coordination of the ATP γ-phosphate may involve participation of residues from adjacent subunits.

The primary enzyme of photosynthetic carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco,[1,2] EC 4.1.1.39), is regulated by Rubisco activase, a chloroplastic enzyme that facilitates the release of sugar phosphates from Rubisco (reviewed in refs *1*−*3*). Without activase, Rubisco is inhibited by tight-binding sugar phosphates that either hinder carbamylation of the activator lysine or block the substrate, ribulose-1,5-bisphosphate, from binding to the carbamylated enzyme. The hydrolysis of ATP by activase is required to convert Rubisco from the tight- to the loose-binding form, presumably by causing conformational changes that reduce the binding affinity of Rubisco for sugar phosphates.

Activase shares a "P-loop" motif with several ATP- and GTPases, including RecA (*4*, *5*), H-*ras* p21 (*6*) and adenylate kinase (*7*). Crystal structures and mutagenesis studies indicate that, in many proteins, the lysine of the P-loop hydrogen bonds to the nucleotide phosphates while the serine/threonine hydroxyl is coordinated to the Mg^{2+} (*4*, *6*, *8*−*10*). In proteins such as RecA (*4*) and adenylate kinase (*11*), an aspartate residue appears to be involved in coordinating the metal ion. This aspartate, which is outside the P-loop, is found at the C-terminal end of a β-strand. This β-strand probably serves as a "scaffold" for the correct positioning of the aspartate residue relative to other key residues in the ATP binding site. On the basis of sequence analysis, including secondary structure prediction, we hypothesized that the species-invariant aspartate at position 231 of activase may be involved in coordination of the Mg^{2+} cofactor. This hypothesis is supported by the identification of D231 in peptides labeled with ATPγbenzophenone, a photoaffinity analogue of ATP in which the photoactive group is adjacent to the γ-phosphate (*12*).

A motif known as the Walker B sequence (cf. ref *13*) is also present near the metal−nucleotide binding sites of several nucleotide binding proteins (*14*, *15*). This motif, a sequence of four hydrophobic residues followed by an aspartate, terminates a β-strand with the aspartate carboxyl group facing the metal−nucleotide binding pocket (*9*, *16*). It has been suggested that this carboxyl either interacts with a metal-bound water or coordinates the metal−nucleotide directly (*9*, *17*).

In most RecA sequences, the Walker B aspartate is replaced by a Gln. This Gln, i.e., Q194 in the *Escherichia coli* enzyme, is thought to couple the nucleotide γ-phosphate with two loops of variable conformation which may interact with the DNA or protein substrate (*4*). In this way, Q194 of RecA may mediate hydrolysis-dependent changes in the conformation of RecA and/or its interaction with its DNA or protein substrate (*4*). Sequence comparisons of tobacco

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[1] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ATPγBP, monoanhydride of ADP with *N*-(4-(benzoyl)-phenylmethyl)phosphoramide; ATPγS, adenosine 5′-[γ-thio]-triphosphate; ANS, 1-anilinonaphthalene-8-sulfonic acid.

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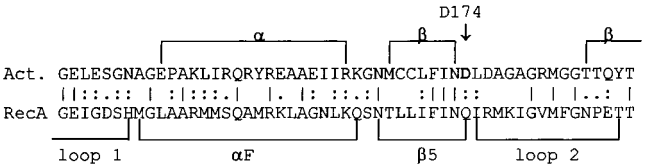

FIGURE 1: Amino acid sequence of tobacco activase (residues 137−189) aligned with *E. coli* RecA (residues 158−210). Secondary structure elements of RecA determined from its crystal structure (*5*) are indicated below its sequence. A secondary structure prediction for activase is indicated above the activase sequence. This prediction was the consensus of predictions made by eight different algorithms [six algorithms implemented by the package ANTHEPROT (*25*); plus PHDsec (*26*); and SSPred (*27*)].

Table 1: Maximal ATPase Activities and Apparent Dissociation Constants for ATP and ADP for Wild-Type and Mutant Activases[a]

enzyme	ATP hydrolysis (U (mg of protein)$^{-1}$)	[ADP] (μM)	[ATP] (μM)	
		0 mM Mg^{2+}	0 mM Mg^{2+}	5 mM Mg^{2+}
WT	0.93	0.40 (0.007)	39.8 (2.77)	7.73 (0.39)
D231R	0	0.24 (0.007)	7.09 (0.87)	4.79 (0.21)
D174Q	0	0.35 (0.019)	6.56 (0.41)	40.2 (2.84)
D174A	0	0.48 (0.027)	9.40 (0.71)	105.8 (15.6)
D174E	0.19	0.70 (0.056)	38.4 (2.71)	84.4 (11.2)

[a] Apparent dissociation constants were determined by ANS fluorescence. The values in parentheses are the standard errors of the mean.

activase with *E. coli* RecA aligns the Walker B aspartate of activase, D174, with Q194 of RecA (Figure 1). Since activase promotes ATP-dependent conformational changes of Rubisco, we were interested in determining if D174 of activase is functionally analogous to Q194 of RecA.

In this report, we describe biochemical studies of recombinant activase enzymes in which mutations were introduced at D231 or D174. The results indicate that both of these residues affect the enzyme's ability to properly coordinate the ATP γ-phosphate for aggregation and catalysis.

MATERIALS AND METHODS

In Vitro Mutagenesis and Protein Purification. A *Sma*I−*Sal*I fragment of wild-type tobacco Rubisco activase cDNA was cloned into M13 phage for site-directed mutagenesis as described (*18*), using the mutagenic oligonucleotide for D231R, 5′ CGT CAC TGG TAA CCG TTT CTC CAC ATT GTA 3′, and for D174QEA, 5′ CCT CTT CAT CAA C(C/G)(A/C) GCT CGA TGC AGG A 3′. The subcloned *Sma*I−*Sal*I fragment was sequenced following mutagenesis to ensure that extraneous mutations were not introduced. Mutant and wild-type proteins were expressed in *E. coli* and purified as described previously (*18*).

Enzyme Analyses. ATPase and Rubisco activation assays were performed as described (*18*). ANS fluorescence titrations (*19*) and intrinsic fluorescence measurements (*18*, *20*) were performed as described using a Hitachi F2000 instrument. Photoaffinity labeling was performed as described previously (*12*, *18*). The photoactive ATP analogue, ATPγbenzophenone (*12*), was generously provided by Drs. B. E. Haley and K. Rajagopalan (University of Kentucky, Lexington, KY).

RESULTS

Recombinant mutant activase proteins were expressed in *E. coli* for purification and study. An arginine residue was substituted for the aspartate at position 231, causing a change in the charge of this residue. The aspartate residue at position 174 was mutated to alanine, as a potentially neutral loss-of-function mutation, and to glutamate and glutamine, as possible conservative substitutions. To test the possibility that G137−T189 of activase and G158−T210 of *E. coli* RecA have a similar tertiary structure (Figure 1), a DNA construct was prepared for expression of a chimeric protein in which this activase segment was replaced by the RecA sequence. Unfortunately, soluble protein corresponding to this chimera could not be detected.

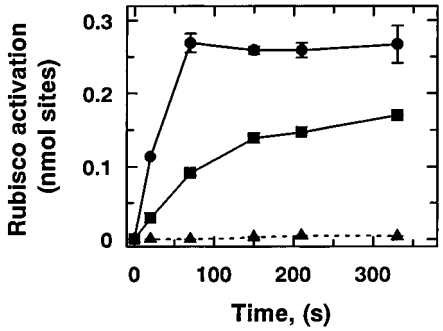

FIGURE 2: Rubisco activation by wild-type activase and the D174E and D231R mutants. Decarbamylated Rubisco complexed with RuBP was incubated at 1.5 mg mL^{-1} in the presence of wild-type (●) or mutant D174E (■) or D231R (▲). At the indicated times, a 15 μL aliquot was removed for determination of Rubisco activity.

The D231R, D174Q, D174A, and D174E mutant proteins were purified by ammonium sulfate precipitation, rate-zonal centrifugation, and anion-exchange chromatography (*18*). That the mutant proteins comigrated with wild-type through a purification regime that exploits a number of physical properties of the enzyme indicated that these mutations caused no major changes in protein structure. One exception was the D231R mutant enzyme, which in two independent experiments migrated further through a sucrose gradient than wild-type enzyme. This result indicated that the D231R mutant may have a larger native quaternary structure than wild-type.

ATP Hydrolysis. The D231R, D174Q, and D174A mutants were unable to catalyze ATP hydrolysis (Table 1). The D174E mutant retained some activity; the maximal activity was approximately 20% of wild-type, and the $S_{0.5}$ was 2-fold higher (i.e., D174E, 254 μM; wild-type, 132 μM). The Hill coefficient of activase for ATP was not affected by substitution of Glu for Asp at 174 (i.e., D174E, 1.47; wild-type, 1.48).

Rubisco Activation. In previous studies, the initial rate of Rubisco activation by activase was directly proportional to the activase-catalyzed rate of ATP hydrolysis (*18*, *21*, *22*). In the present study, the D231R mutant, which had no ATPase activity, was unable to activate Rubisco. The D174E enzyme exhibited residual Rubisco activation activity, comparable to the activity of ATP hydrolysis (Figure 2). For example, the initial rate of Rubisco activation was approximately 26% of the wild-type enzyme. After 5 min, the level of activation attained by the mutant was approximately 62% of the wild-type.

Binding of ATP. Titrations of activase−ANS with ATP in the presence or absence of 5 mM $MgCl_2$, and with ADP in the absence of Mg^{2+}, were used to obtain apparent dissociation constants for ATP and ADP (Table 1). In the absence of Mg^{2+}, wild-type enzyme had a higher apparent

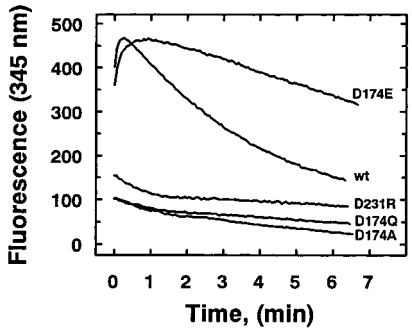

FIGURE 3: Intrinsic fluorescence of wild-type and mutant activases. Time course of fluorescence after the addition of 0.2 mM ATP at zero time.

affinity for ADP than ATP. With 5 mM Mg^{2+}, the affinity for ATP increased markedly. Wang and Portis (*19*) reported similar values for ATP in the presence and absence of Mg^{2+} and showed that when Mg^{2+} is present, the apparent dissociation constants for ADP and ATP are similar. Mutation of Asp231 to Arg mimicked the effect of Mg^{2+} on wild-type enzyme, increasing the apparent affinity of the enzyme for ATP in the absence of Mg^{2+} to a value very similar to that of wild-type in the presence of Mg^{2+}. Similarly, the level of incorporation of the ATP analogue, ATPγbenzophenone, into the activase polypeptide was considerably greater for the D231R mutant than for wild-type in the absence of Mg^{2+}, but not in its presence (data not shown).

Mutation of Asp174 to Gln or Ala increased the apparent affinity for ATP in the absence of Mg^{2+}, but decreased it in the presence of Mg^{2+} (Table 1). Interestingly, the apparent affinity for ATP of the D174E mutant was nearly identical to wild-type in the absence of Mg^{2+} but was more than 10-fold lower than wild-type when Mg^{2+} was present.

Aggregation. The intrinsic fluorescence of activase increases when both ATP and Mg^{2+} are added and decays as ATP is hydrolyzed (*18, 20*). Analysis of the D231 and D174 mutants showed that only the D174E mutant enzyme exhibited an increase in intrinsic fluorescence upon addition of ATP and Mg^{2+} (Figure 3). The kinetics of the fluorescence decay was slower for the D174E mutant than for wild-type. Mixing experiments with wild-type and the D231R and D174Q mutants showed that the presence of the mutant protein inhibited the increase in intrinsic fluorescence that accompanies addition of ATP to wild-type enzyme (data not shown).

Mixing Experiments. When mutants lacking ATPase activity were mixed with wild-type enzyme, the wild-type/mutant mixtures had lower ATPase activity than the wild-type enzyme alone (Figure 4). The extent of inhibition depended on the individual mutant and the ratio of mutant to wild-type enzyme. The D231R mutant strongly inhibited wild-type enzyme, reducing activity by almost 80% at a mutant/wild-type ratio of 4:1 (Figure 4A). The D174Q mutant had a weaker effect, giving 40% inhibition at this same ratio (Figure 4B). In contrast, the mutant with residual ATPase activity, D174E, rather than inhibiting wild-type enzyme ATPase activity, stimulated activity to a level that was the same or just slightly higher than the sum of the component activities (Figure 4B). A stimulation of specific activity with increasing protein concentration has been described for the wild-type enzyme (*23*).

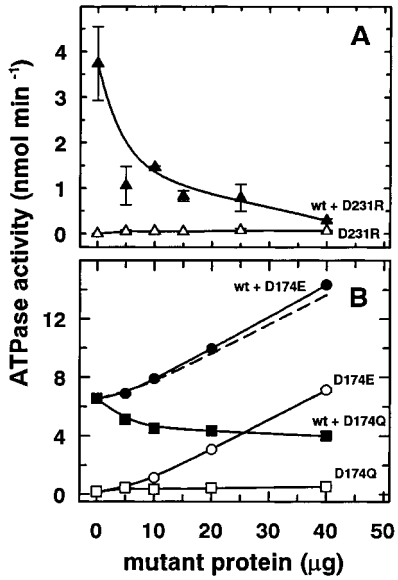

FIGURE 4: Effect of mutant activase on the ATPase activity of wild-type activase. (A) ATPase activity was determined after mixing the indicated amounts of the D231R mutant with either 5 μg of wild-type (wt) activase (▲) or buffer (△). (B) ATPase activity was determined after mixing the indicated amounts of the D174E (●, ○) or D174Q (■, □) with either 10 μg of wild-type activase (●, ■) or buffer (○, □)). The dashed line indicates the arithmetic addition of the activities of D174E and wild-type separately.

DISCUSSION

ATP and Cofactor Binding. There is mounting evidence that the interaction of ATP with activase can be resolved into at least three distinct phases. The first phase is the initial binding of ATP/Mg^{2+}. This binding can be measured directly by photoaffinity labeling (*12*, *18*), or indirectly through changes in ANS fluorescence (*19*). With both of these methods, hyperbolic binding kinetics with binding constants in the 5−10 μM range are observed. The initial binding of ATP/Mg^{2+} is quite rapid, indicating that this first binding step is essentially instantaneous, and noncooperative.

In the present study, the apparent affinity of wild-type activase for ADP was much higher than for ATP in the absence of Mg^{2+}. This result confirmed the findings of Wang and Portis (*19*) and indicated that the γ-phosphate of ATP weakens the binding of the adenine nucleotide. However, when the Mg^{2+} cofactor was present, the apparent affinity for ATP increased to a level similar to that for ADP. Thus, as in RecA (*4*) and other ATP- and GTPases (*6, 8, 11, 17*), Mg^{2+} apparently coordinates the γ-phosphate of ATP, possibly through interaction with an aspartate residue.

To examine the role of D231 and D174 in the initial binding of ATP/Mg^{2+} by activase, we mutated these residues and then measured the interaction of cofactor and nucleotides with ANS−activase. The D231R, D174Q, and D174A mutants all had higher apparent affinity for ATP in the absence of Mg^{2+}, as compared to wild-type enzyme. By contrast, the apparent affinity for ATP of the D174E enzyme was the same as for wild-type. These results suggested that replacement of the negatively charged aspartate at either position 174 or 231, with an uncharged or positive residue, allowed greater access to the site by the negatively charged nucleotide. Improved access of ATP to its binding site is consistent with the possibility that there are normally ionic

interactions between the D231 and D174 residues and the Mg^{2+} cofactor. Upon addition of Mg^{2+}, the affinity for ATP of the wild-type and D231R proteins increased, but decreased for the D174Q, D174A, and D174E mutants. A possible interpretation of these results is that for the D231R mutant, Mg^{2+} is still able to interact with other residues of the protein, possibly D174, in a manner that facilitates ATP binding, whereas the same is not true of mutants altered at D174.

Aggregation and Hydrolysis. The second phase of the activase−ATP/Mg^{2+} interaction is aggregation of the enzyme into higher-ordered oligomers. This phase of the interaction is accompanied by an increase in the intrinsic fluorescence of activase (*18*, *20*), signaling a change in the environment of a tryptophan residue, probably Trp250 (*18*), to a more hydrophobic condition. This phase is much slower than the initial binding of ATP and Mg^{2+} to the enzyme, requires higher ATP concentrations, and has a sigmoidal rather than hyperbolic response to ATP concentration (*20*). In the present study, the increase in intrinsic fluorescence that normally occurs upon addition of ATP/Mg^{2+} only occurred for the D174E mutant. That this response was not observed for the D174Q, D174A, or D231R mutants indicates that, though the mutant subunits bound ATP, they did not aggregate.

The third and final phase of the interaction between ATP/Mg^{2+} and activase is ATP hydrolysis. Three lines of evidence indicate that this phase is preceded by aggregation and that each reaction cycle involves aggregation and disaggregation. First, after the addition of ATP, there is a lag of about 2 min before a maximal rate of hydrolysis is observed. This lag is very similar to the time required for aggregation (*20*). Second, ATPase specific activity depends on the concentration of activase (*23*), as does the rate of increase in intrinsic fluorescence (*20*). Third, intrinsic fluorescence (i.e, aggregation) increases to a constant level upon addition of the nonhydrolyzable analogue, ATPγS (*18*, *20*), but declines upon hydrolysis of the γ-phosphate of ATP. Taken together, these results suggest that hydrolysis of ATP causes disaggregation and that the product ADP must be displaced before re-aggregation can occur.

In the present study, the D174Q, D174A, and D231R mutants did not appear to aggregate and were incapable of catalyzing ATP hydrolysis, even though they bound ATP. Thus, these mutations affected the enzyme's ability to undergo the conformational changes that commit the enzyme first to aggregation and then to catalysis. These changes in conformation may involve proper coordination of the Mg^{2+} cofactor, which may have been compromised in the mutants. Interestingly, the least potentially compromised of the mutants, D174E, both aggregated and hydrolyzed ATP, albeit at much slower rates than wild-type. The slower rate at which intrinsic fluorescence of the D174E mutant declined from its maximum was consistent with its slower rate of ATP hydrolysis. The slower rate at which intrinsic fluorecence increased upon addition of ATP suggests that the rate of subunit aggregation may be rate determining for ATP hydrolysis.

Mixing experiments provided further insight into the roles of D231 and D174 in aggregation and ATP hydrolysis. Mutants that did not aggregate or hydrolyze ATP inhibited wild-type enzyme activity and prevented wild-type enzyme from aggregating. Presumably, mutant activase subunits interact with wild-type activase as lower-ordered oligomers, an interaction that prevents higher-ordered aggregates from forming and catalyzing ATP hydrolysis. Previous mixing experiments with the ATPase-deficient mutant K247R showed that this mutant inhibits wild-type activase from activating Rubisco (*24*) in a manner very similar to that presented here. Addition of K247R mutant caused a 75% reduction in Rubisco activation by wild-type activase, whereas the D231R mutant caused almost complete inhibition of wild-type ATPase activity and the D174A caused a 40% reduction in ATPase activity. Thus, the maximum inhibition of wild-type activase by the ATPase-deficient mutants depended on the nature and site of the lesion and not simply on the absence of activity.

The precise mechanism by which the subunits of activase aggregate to form a catalytically competent enzyme is unknown. One possibility is that residues necessary for catalysis are contributed by neighboring subunits. Alternatively, the effect could be intramolecular, involving an aggregation-induced conformational change that alters the positioning of essential residues within the ATP binding site of an individual subunit. The results of this and previous (*18*, *20*) studies suggest that precise coordination of the γ-phosphate, probably through proper ligation of Mg^{2+}, is necessary for aggregation to occur. Our results confirm previous results (*20*) which suggested that only the aggregated state is competent for ATP hydrolysis and Rubisco activation. While the K247R mutant (*24*) and each of the ATPase-deficient mutants described here bind ATP, this "coarse" binding is not followed by the more precise coordination required for the subsequent steps in the catalytic mechanism. The inhibitory effect of the mutants on wild-type activity suggests that D174, D231, and K247 affect the precise coordination of the γ-phosphate of ATP on a neighboring subunit. While accounting for the high degree of cooperativity of wild-type Rubisco activase, it remains to be determined if this effect is conformational or involves completion of the ATP binding site by an adjacent subunit.

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