Concurrent Chaperone and Protease Activities of ClpAP and the Requirement for the N-terminal ClpA ATP Binding Site for Chaperone Activity*

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The Journal of Biological Chemistry Vol. 274, No. 27, Issue of July 2, pp. 19316–19322, 1999

ClpA, a member of the Clp/Hsp100 family of ATPases, is both an ATP-dependent molecular chaperone and the regulatory component of ClpAP protease. We demonstrate that chaperone and protease activities occur concurrently in ClpAP complexes during a single round of RepA binding to ClpAP and ATP-dependent release. This result was substantiated with a ClpA mutant, ClpA(K220V), carrying an amino acid substitution in the N-terminal ATP binding site. ClpA(K220V) is unable to activate RepA, but the presence of ClpP or chemically inactivated ClpP restores its ability to activate RepA. The presence of ClpP simultaneously facilitates degradation of RepA. ClpP must remain bound to ClpA(K220V) for these effects, indicating that both chaperone and proteolytic activities of the mutant complex occur concurrently. ClpA(K220V) itself is able to form stable complexes with RepA in the presence of a poorly hydrolyzed ATP analog, adenosine 5’-O-(thiotriphosphate), and to release RepA upon exchange of adenosine 5’-O-(thiotriphosphate) with ATP. However, the released RepA is inactive in DNA binding, indicating that the N-terminal ATP binding site is essential for the chaperone activity of ClpA. Taken together, these results suggest that substrates bound to the complex of the proteolytic and ATPase components can be partitioned between release/reactivation and translocation/degradation.

The Clp/Hsp100 ATPase family of proteins are highly homologous and ubiquitous in nature (1). All members contain one or two very conserved ATP binding domains and function as ATP-dependent molecular chaperones and/or as regulatory components of energy-dependent proteases (1, 2). ClpA of Escherichia coli is a well studied example. It carries out the chaperone function of DnaJ and DnaK in the activation of the latent DNA binding activity of RepA, the plasmid P1 DNA replication initiator protein, by converting inactive dimers to active monomers (3, 4). ClpA also protects proteins, including firefly luciferase and RepA, from irreversible heat inactivation in vitro (3). In addition, ClpA targets specific proteins for degradation by ClpP, a peptidase that alone is unable to degrade proteins, in the presence of a poorly hydrolyzed ATP analog, adenosine 5’-O-(thiotriphosphate), and to release RepA upon exchange of adenosine 5’-O-(thiotriphosphate) with ATP. However, the released RepA is inactive in DNA binding, indicating that the N-terminal ATP binding site is essential for the chaperone activity of ClpA. Taken together, these results suggest that substrates bound to the complex of the proteolytic and ATPase components can be partitioned between release/reactivation and translocation/degradation.

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¶ This paper is available on line at http://www.jbc.org

1 The contribution of each of the two ATP binding domains of ClpA to oligomerization, ATP hydrolysis, and proteolysis has been addressed by studies of ClpA proteins with mutations at the conserved lysine in one or the other of the ATP binding sites (30, 31). The N-terminal site is involved in the assembly of ClpA hexamers, and the C-terminal site is involved in ATP hydrolysis and degradation of proteins. A functional C-terminal site is not required for propeptide degradation, a reaction whose nucleotide requirement is met by nonhydrolyzable ATP analogs (31).

From studies of the mechanism of the chaperone activity of ClpA and the proteolytic activity of ClpAP, the pathways of protein remodeling and degradation have emerged. During RepA activation, a stoichiometric complex of ClpA hexamers and RepA dimers forms in a reaction requiring nonhydrolyzable ATP analog (4). On each cycle of the nonhydrolyzable ATP analog with ATP, RepA dimers are converted to monomers that can bind with high affinity to RepA-specific DNA binding sites. A single cycle of RepA binding to ClpA and ATP-dependent release from ClpA is sufficient to activate RepA. For protein degradation, ClpA–ClpP substrate complexes assemble in the presence of a nonhydrolyzable ATP analogs.
analog, by either ClpA-substrate complexes binding ClpP or ClpA-ClpP complexes binding substrates (32). Then in a reaction dependent on ATP hydrolysis, ClpA translocates specific substrates to ClpP for subsequent degradation. Degradation of some substrates, including RepA and α-casein, can be accomplished in a single round of substrate binding to ClpAP and ATP-dependent release of products that are identical to the steady state products.

The current model for the role of ATPase components or domains in ATP-dependent proteolysis proposes that the chaperone activity functions early in the pathway of degradation by unfolding or remodelling specific substrates and irreversibly damaged proteins to allow substrate translocation to the proteolytic chamber for proteolysis (1, 33). The model also proposes that the ATPase components function in regulating proteolysis by remodelling and reactivating less severely damaged proteins and some specific substrates. Thus one prediction of this model is that the chaperone activity is manifest when the ATPase component is assembled in the proteolytic complex. A second prediction is that the chaperone activity is an essential intermediate step during proteolysis. The results presented in this study provide support for both of these predictions.

We demonstrate that ClpA, while in a complex with ClpP, is able to carry out concurrently chaperone and proteolytic activities. We also found, by characterizing ClpA derivatives with mutations in each of the two ATP binding sites, that the N-terminal site is essential for the chaperone activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, ATPγS,1 and Triton X-100 were obtained from Roche Molecular Biochemicals. [γ-32P]ATP was from ICN Pharmaceuticals, Inc. Sepharose S-100 and S-200 high resolution gel filtration media were obtained from Amersham Pharmacia Biotech.

**Proteins and DNA**—P1 RepA (34), ClpA (35), ClpA mutant proteins (30), ClpP (35), and ClpP mutated in its serine active site (S111A) (36) were purified as described. Chemical inactivation of ClpP with succinyl-Leu-Tyr-chloromethyl ketone was as described (32). [3H]-Labeled oriP1 plasmid DNA (4), [3H]-labeled linear oriP1 DNA (37), [3H]RepA, and [α-32P]-casein (32) were prepared as described. Protein concentrations are expressed as molar amounts of RepA dimers, ClpA hexamers, ClpP tetradecamers, and α-casein monomers.

**RepA Activation Assay**—Reaction mixtures contained (in 20 μl) Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM EDTA), 1 mM ATP, 100 μg/ml bovine serum albumin, 1.4 pmol of ClpA, and 0.03 pmol of RepA, unless indicated otherwise. Reaction mixtures with ClpA mutant derivatives contained 4 mM ATP. After 10 min at 23 °C, unless indicated otherwise, the mixtures were chilled to 0 °C. One μg of calf thymus DNA and 40 pmol of [3H]oriP1 plasmid DNA or [3H]oriP1 linear DNA were added. After 5 min at 0 °C, the mixtures were filtered through nitrocellulose filters, and the retained radioactivity was measured.

**RepA Degradation Assay**—Reaction mixtures were assembled in 50 μl of Buffer B (20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 100 mM KC1, 5 mM dithiothreitol, 0.005% Triton X-100 (v/v)) containing 4 mM ATP. 0.8 pmol of ClpA or ClpA mutant proteins, 4.7 pmol of [3H]RepA, and 0.3 pmol of ClpP. The mixtures were incubated at 37 °C for 15 min unless indicated otherwise. Trichloroacetic acid was added to 20% (w/v), and RepA degradation was quantitated by measuring acid-soluble radioactivity.

**Gel Filtration Chromatography of RepA-ClpA Complexes**—To isolate RepA-ClpA complexes, reaction mixtures (70 μl) containing 500 pmol of [3H]RepA, 100 pmol of ClpA or ClpA(K220V), and 1 mM ATPγS in Buffer A containing 5% (v/v) glycerol were incubated for 15 min at 23 °C. The reaction mixtures were then loaded onto a Sephacryl S-200 HR column (0.7 cm x 7 cm) equilibrated with Buffer A containing 5% glycerol and 1 mM ATP. Fractions (125 μl) of the pooled material were incubated in a 70-μl reaction mixture with 10 mM ATP for 10 min at 23 °C and applied onto a Sephacryl S-200 HR column (0.7 cm x 7 cm) equilibrated with Buffer A containing 5% glycerol and 1 mM ATP. Fractions (125 μl) were collected, and radioactivity in each fraction was measured.

**RESULTS**

**ClpA(K220V) Lacks Chaperone Activity but Destroys RepA in Conjunction with ClpP**—Our first evidence to support the hypothesis that the chaperone and proteolytic activities of ClpAP are concurrent came from the study of a ClpA mutant. This mutant, ClpA(K220V), has a valine substitution for the conserved lysine in the N-terminal ATP binding domain. Using activation of specific DNA binding by plasmid P1 RepA as a measure of chaperone activity, we found that ClpA(K220V) retained <5% of the wild-type ClpA ability to activate RepA for DNA binding (Fig. 1A).

Surprisingly, when we examined this chaperone defective ClpA mutant for its ability to promote RepA degradation by ClpP, we found that ClpA(K220V)P degraded RepA (Fig. 1B). The rate of RepA degradation by ClpA(K220V)P was slightly faster than by ClpA wt/P. Consistent with its ability to degrade RepA, ClpA(K220V)P degraded α-casein at 60% of the rate of wild-type ClpAP (Table III).

Because ClpA(K220V) was able to function in protein degradation, we wanted to test the efficiency of this mutant in translocating substrates to ClpP. We used a protocol that measures transfer of substrates from ClpA binding sites to ClpP without degradation (32). Using proteolytically inactive variants of ClpP, obtained by either chemical modification or site-directed mutagenesis, [3H]RepA-ClpA(K220V)P complexes were formed in the presence of ATPγS. ATP was added in a 10-fold molar excess to ATPγS to facilitate transfer of RepA to the inactive ClpP. The reaction mixtures were treated with 1 mM NaCl to dissociate ClpA from ClpP, and then ClpP antibody was added to immunoprecipitate ClpP and any associated [3H]RepA. Last, radioactivity in the immunoprecipitates was

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1 The abbreviations used are: ATPγS, adenosine-5′-O-(3-thiotriphosphate); wt, wild type.
When ClpA(K220V) was used in this assay, we found that RepA was transferred to inactive ClpP (Fig. 2, columns 1 and 2). ATP was required for the translocation (Fig. 2, column 3), and the extent of the reaction was similar to that seen with wild-type ClpA (Fig. 2, columns 1 and 2).

ClpA(K220V)P Is Able to Concurrently Activate and Degrade RepA—The observation that ClpA(K220V) was defective in chaperone activity but functional in proteolysis with ClpP was in apparent contradiction to the proposal that substrate unfolding or remodeling by the ATPase component is a prerequisite step in the pathway of degradation. The fact that ClpA(K220V) has the ability to transfer substrates to ClpP implied that ClpA(K220V) also has the ability to unfold substrates. The simplest explanation for this seeming discrepancy was that the presence of ClpP restored the chaperone activity of ClpA(K220V). To test this, we assayed RepA activation by ClpA(K220V) in the presence of ClpP. RepA-ClpA(K220V) complexes were assembled in the presence of ATPγS, α-casein was added to limit the reaction to a single cycle of RepA binding, and last, excess ATP was added. Under these conditions, we found that ClpA(K220V) was able to activate RepA in the presence of ClpP, but not in its absence (Fig. 3A). ClpA(K220V) also activated RepA when chemically inactive ClpP (ClpAP, ClpA(Pin), or ClpA(Pin)), was substituted for wild-type ClpP. In control experiments with wild-type ClpA, RepA was activated by ClpA alone, ClpAP, or ClpAP(Pin) (Fig. 3B). Thus, these results show that the chaperone defect of ClpA(K220V) is reversed by the interaction of ClpA(K220V) with ClpP.

To determine whether ClpP was necessary throughout the activation reaction or only to promote a functional conformation in RepA-ClpA(K220V) complexes, we assembled [3H]RepA-ClpA(K220V) complexes in the presence of ATPγS. Next the complexes were treated with 1 M NaCl, a treatment known to dissociate ClpA-ClpP but not ClpA-RepA (32). After a short incubation, ATP was added in a large excess over ATPγS, and activated RepA was measured. The results showed that ClpA(K220V), unlike ClpA, was not able to activate RepA after dissociation from ClpP (Fig. 4A). Control experiments showed that the NaCl treatment did not disrupt RepA-ClpA(K220V) or RepA-ClpA complexes (data not shown). RepA degradation from both RepA-ClpA(K220V)P and RepA-ClpAP complexes was abolished by NaCl treatment as expected, because ClpP was dissociated by the treatment (Fig. 4B). When NaCl was added after α-casein and ATP, ClpA(K220V)P complexes activated and degraded RepA, indicating that functional RepA-ClpA(K220V)P complexes were formed under these conditions (data not shown).

Thus, the initially surprising result that ClpA(K220V) lacks chaperone activity but has proteolytic activity with ClpP is explained by the ability of ClpP to overcome the chaperone defect of ClpA(K220V), very likely by stabilizing a conformation of ClpA that is essential for the chaperone activity. Thus, these results support the proposal that protein unfolding or remodeling needed for presentation of substrates to the proteolytic component occurs by a mechanism similar to that of protein remodeling involved in the chaperone activity.
Characterization of ClpA(K220V)—We further characterized ClpA(K220V) with the expectation that by understanding the step in the chaperone pathway that was blocked, we would determine whether ClpA(K220V) could assemble into hexamers, we incubated ClpA(K220V) with or without ATPγS and analyzed the samples by size exclusion column chromatography. ClpA(K220V), like ClpA, eluted as expected for a hexamer of 84-kDa protomers in the presence of nucleotide (Fig. 6). Electron microscopy of ClpA(K220V) showed a preponderance of hexameric rings in the presence of ATPγS. Thus, ClpA(K220V) is not blocked in assembly. Measurement of ATPase activity showed that ClpA(K220V) hydrolyzed ATP at about 60% that of the rate of wild-type ClpA and was stimulated 2-fold by ClpP(in) (Table 1).

To investigate whether ClpA(K220V) hexamers form stable complexes with RepA, we incubated [3H]RepA and ClpA(K220V) with ATPγS and examined the complexes by gel filtration chromatography. About 15% of the RepA eluted in the excluded volume with ClpA(K220V) as expected, because a 5:1 ratio of RepA to ClpA(K220V) was used (Fig. 7). With wild-type ClpA, similar results were obtained (Fig. 7 and Ref. 4). Thus, ClpA(K220V) forms relatively stable complexes with RepA.

Because it is known that ATP promotes the release of RepA from RepA-ClpA complexes in a form active for DNA binding (4), we wanted to know if ClpA(K220V) was defective in its ability to release RepA. We isolated [3H]RepA-ClpA(K220V)-ATPγS complexes, incubated them with an excess of ATP over ATPγS, and resolved free RepA from ClpA(K220V)-RepA complexes by gel filtration. We found that RepA was freed from ClpA(K220V) and eluted in the partially included volume (Table II). To see whether RepA released from RepA-ClpA(K220V) complexes was in its active or inactive form, we incubated a portion of the [3H]RepA-ClpA(K220V)-ATPγS complexes (containing 0.16 pmol RepA) with or without the addition of wild-type ClpA (1.4 pmol) and a 10-fold molar excess of ATP over ATPγS. After 10 min of incubation at 23 °C, activated RepA was measured as described under “Experimental Procedures.” RepA from RepA-ClpA(K220V) complexes was inactive for DNA binding (less than 0.5 fmol of oriP1 DNA bound) but was activated upon

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2 F. Beuron and M. Maurizi, unpublished data.
addition of ClpA (8 fmol of oriP1 DNA bound). As a control, RepA from RepA-ClpA(wt) complexes was active without the addition of ClpA (12 fmol of oriP1 DNA bound). Therefore, ClpA(K220V), like wild-type ClpA, is able to bind RepA and to release RepA in an ATP-dependent reaction. However, the RepA released from ClpA(K220V) is inactive, demonstrating that the defect in ClpA(K220V) is its inability to carry out the remodeling step. Finally, RepA does not appear to be modified while in complex with ClpA(K220V) because it can be activated by wild-type ClpA.

**Characterization of the Chaperone and Protease Activities of Other ClpA Mutants**—Two other ClpA mutants with different amino acids substituted for K220 were tested for chaperone activity. ClpA(K220R) was deficient in RepA activation, but the defect was less severe than that of ClpA(K220V) (Fig. 8A). RepA was degraded by ClpA(K220R)P at about 25% that of the rate of wild-type ClpAP (Fig. 8B). ClpA(K220Q), which is defective in assembly, ATP hydrolysis, and α-casein degradation (30) lacked chaperone activity (Fig. 8A) and did not promote RepA degradation with ClpP (Fig. 8B).

We also tested ClpA proteins mutated in the C-terminal ATP binding site. ClpA(K501R) and ClpA(K501Q) retained 60–70% of the wild-type chaperone activity (Fig. 8C), although they had <10% of the wild-type ATPase activity (30). With ClpP, these two mutants degraded RepA at 15–25% of the wild-type rate (Fig. 8D). Thus a functional N-terminal ATP binding site, but not a C-terminal site, is essential for chaperone activity.

**DISCUSSION**

The results presented here point out an important new element in the model of energy-dependent proteolysis. They demonstrate that both chaperone and protease activities can occur concurrently in ClpAP complexes. Thus the fate of a substrate...
to be degraded instead of refolded is not simply a consequence of a proteolytic component associating with an ATPase component. Our working model for ClpAP is shown in Fig. 9. First, RepA

\[ \text{ClpA} + \text{ATP}[\gamma-S] \]

binds ClpA in a reaction requiring ATP. Exchange of the bound ATP with ATP leads to substrate unfolding and remodeling by ClpA, and subsequently, some RepA is released as active monomers, and some is translocated to ClpP for degradation. In the absence of ClpP, RepA binds ClpA in a reaction requiring ATP. Following ATP exchange, RepA is unfolded, remodeled, and released as active monomers. Although it is known that one ClpA/ClpP14 interacts with one RepA dimer, it remains to be determined if one RepA monomer is degraded and one is activated from each RepA/ClpAP complex.

In our working model, the role of Clp ATPases in proteolysis is 3-fold: (i) recognizing and binding substrates (10, 32, 38), (ii) unfolding substrates, and (iii) translocating substrates to the proteolytic chamber of ClpP (32). Protein unfolding as a step in the pathway of degradation has yet to be directly demonstrated but has been postulated based on the finding that Clp ATPases have chaperone activity (3, 10) and on the structural analysis of ClpP showing that the axial pores leading to the ClpP proteolytic chamber are only large enough to allow passage of a \( \beta \) strand or an \( \alpha \) helix (29). Very likely, the chaperone activities of Clp ATPases also involve unfolded substrate intermediates. Thus, RepA may be released in an unfolded state that spontaneously refolds into an active monomer. Consistent with this, RepA is rapidly activated by treatment with guanidine-HCl followed by dilution of the denaturant. The observation that a ClpA mutant, which is by itself devoid of chaperone activity, is able to facilitate proteolysis by ClpP initially appeared to contradict the working model. However, our finding that ClpP overcomes the chaperone defect of the mutant ClpA supports the proposal that substrate unfolding needed for presentation of substrates to the proteolytic component occurs by a mechanism similar to that of protein remodeling involved in the chaperone activity.

Cryo-electron microscopy has shown that the ClpAP complex contains two internal aqueous chambers in addition to the proteolytic cavity in ClpP. One chamber is present between the

![FIG. 9. Model of concurrent activation and degradation of RepA by ClpAP. See text for discussion.](image)

| TABLE III | Summary of activities of ClpA mutants |
|-----------|--------------------------------------|
| ClpA      | RepA activation | RepA degradation | \( \alpha \)-Casein degradation | ClpA assembly | ClpA-mediated translocation to ClpP | ATPase activity |
| ClpA(wt)  | 100 | 100 | 100 | + | 100 | 100 |
| ClpA(R220V) | 0 | 115 | 60 | + | 130 | 60 |
| ClpA(R220R) | 5 | 25 | 32 | + | 60 | 60 |
| ClpA(R220Q) | 0 | 0 | 0 | - | NT | 0 |
| ClpA(R220T) | NT | NT | 46(19) | + | (—) | NT |
| ClpA(R501R) | 60 | 25 | 6 | + | NT | 70(15) |
| ClpA(R501Q) | 70 | 14 | 2 | + | NT | 9 |
| ClpA(R501T) | NT | NT | 0 | + | NT | 0 |

* Assays were carried out as described under “Experimental Procedures.”
* Data from Singh and Maurizi (30).
* NT, not tested.
* Data from Seol et al. (31).
* Values in parenthesis indicate results obtained with 0.5 mM ATP (31).

3 M. Pak and S. Wickner, unpublished observation.

4 S. Wickner, unpublished observation.
two ATPase domains of ClpA, and another is formed by the
junction of the proximal ATPase domain and ClpP (28). Elec-
tron micrographs of RepA-ClpAP complexes indicate that large
conformational changes occur within the complex when ATP is
added. These changes appear to increase the sizes of the
internal chambers and to widen the narrow channels connect-
ing the ClpA chambers to the ClpP chamber. Such changes are
consistent with our current working model for the mechanism
of ClpAP and suggest a means by which unfolded substrates
can be translocated to the proteolytic chamber without expo-
sure to the surrounding medium.

It has recently been shown that ClpX is able to function as a
chaperone while associated with ClpP (39). However, the
results with ClpXP differ from those presented here with ClpAP in
that the substrate for the chaperone activity is distinct from
the substrate for proteolytic activity. Jones et al. (39) found
that saturating levels of ClpP do not inhibit the ability of ClpX
to perform its chaperone function of promoting MuA-dependent
Mu transposition in vitro, although free MuA transposase is
degraded by ClpXP. In fact, ClpXP stimulates Mu transposition
2–3-fold without degrading MuA in the transpososome. In this
case it is likely that the two substrates, MuA-DNA and free
MuA transposase, are recognized differently by ClpXP and are
consequently remodeled or degraded, respectively. Altogether,
the studies from ClpAP and ClpXP suggest that concurrent
chaperone and proteolytic activities may be a general function
of proteolytic systems.

Our results showing that substitution of a valine for lysine at
residue 220 of ClpA eliminates the ability of ClpA to remodel
RepA imply that ATP binding in the first domain is necessary
for the conformational changes of ClpA associated with protein
modeling. The restoration of ClpA(K220V) chaperone activity
and the increase in ATPase activity in the presence of ClpP
suggest that interactions between ClpA(K220V) and ClpP lead
to critical conformational changes in ClpA(K220V) that over-
come the barrier in RepA activation. The elimination of
ClpA(K220V) chaperone activity upon dissociation of ClpP from
RepA-ClpA(K220V)P complexes by NaCl treatment further
suggests that ClpP facilitates and stabilizes the formation of a
conformation necessary for chaperone activity. Additional
evidence for ClpP-mediated structural changes in ClpA as a
general aspect of ClpAP interactions is provided by the observa-
tion that ClpP is able to stimulate the ATPase activities of other
ClpA mutants in the first ATP binding site (30, 31).

Table III summarizes the properties ClpA derivatives con-
taining mutations in the N-terminal and C-terminal ATP bind-
ing sites. Previous work had shown that mutations in the first
ATP binding domain can interfere with ClpA hexamer assem-
by, and mutations in the second domain can impair ATPase and
protease activities (30, 31). The present results show that the
first ATP binding site of ClpA is necessary for RepA activation,
and the second site contributes only minimally. Ulti-
mately, optimal chaperone activity of ClpA and proteolytic
activity of ClpAP require some contribution from both ATP
binding domains. Similar studies of Hsp104 derivatives con-
taining mutations in the two nucleotide binding sites demon-
strated that the first domain of Hsp104 contributes to the ATP
hydrolytic activity, and the second domain contributes to
assembly (40). This reversal of the roles does not indicate any
novel distinction between ClpA and Hsp104. As seen for ClpA,
each domain of Hsp104 appears to have distinct ac-
tivity, both domains have overlapping functions that are criti-
ical for optimal activity.

Altogether, our work demonstrates the versatility of
toes to function simultaneously in substrate remodeling and
degradation. The present work also shows the conformational
changes induced by ATP binding or hydrolysis in the N-termi-
al ATP binding domain constitute a crucial step toward ClpA
chaperone activity.

Acknowledgments—We thank Hua-Yong Yang for preparation of
ClpA(K220V) protein and preliminary observations and Susan Gottes-
man for helpful discussions.

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