Chaperonin-Facilitated Refolding of Ribulosebisphosphate Carboxylase and ATP Hydrolysis by Chaperonin 60 (groEL) Are K^+ Dependent

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ABSTRACT: Both the chaperonin- and MgATP-dependent reconstitution of unfolded ribulosebisphosphate carboxylase (Rubisco) *and* the uncoupled ATPase activity of chaperonin 60 (groEL) require ionic potassium. The spontaneous, chaperonin-independent reconstitution of Rubisco, observed at 15 but not at 25 °C, requires no K^+ and is actually inhibited by chaperonin 60, with which the unfolded or partly folded Rubisco forms a stable binary complex. The chaperonin-dependent reconstitution of Rubisco involves the formation of a complex between chaperonin 60 and chaperonin 10 (groES). Formation of this complex almost completely inhibits the uncoupled ATPase activity of chaperonin 60. Furthermore, although the formation of the chaperonin 60–chaperonin 10 complex requires the presence of MgATP, hydrolysis of ATP may not be required, since complex formation occurs in the absence of K^+. The interaction of chaperonin 60 with unfolded or partly folded Rubisco does not require MgATP, K^+, or chaperonin 10. However, discharge of the complex of chaperonin 60–Rubisco, which leads to the formation of active Rubisco dimers, requires chaperonin 10 and a coupled, K^+-dependent hydrolysis of ATP. We propose that a role of chaperonin 10 is to couple the K^+-dependent hydrolysis of ATP to the release of the folded monomers of the target protein from chaperonin 60.

Since the in vitro folding of many purified proteins occurs in the complete absence of other cellular factors and without the input of energy, it is generally thought to proceed in vivo by the same spontaneous mechanism. Recently, however, a number of accessory proteins have been implicated in the in vivo folding process [reviewed by Fischer and Schmid (1990)]. These accessory proteins may be grouped into two classes, depending upon their requirement for ATP.

In the ATP-independent class are the protein isomerases, protein disulfide-isomerase (Freedman, 1989) and prolyl cis–trans isomerase (Fischer et al., 1984; Fischer & Schmid, 1990). These protein isomerases accelerate the rate-determining step in the folding of some other proteins by molecular mechanisms that are understood in principle, if not in detail.

Another class of accessory proteins, the so-called chaperonins (Ellis & Hemmingsen, 1989), function in an ATP-dependent manner. These include the ubiquitous chaperonin protein chaperonin 60 (cpn60)[1] from bacteria (previously designated groEL) (Hendrix, 1979; Hohn et al., 1979), chloroplasts (Hemmingsen et al., 1988), and mitochondria (McMullin & Hallberg, 1988; Picketts et al., 1989). A second chaperonin protein, chaperonin 10 (cpn10) (previously designated groES), from bacteria has been described (Chandrasekhar et al., 1986). There is both genetic (Tilly & Georgeopoulos, 1982; Zweig & Cummings, 1973; Goloubinoff et al., 1989a; Kusukawa et al., 1989; Van Dyk et al., 1989) and biochemical (Chandrasekhar et al., 1986; Goloubinoff et al., 1989b) evidence that cpn60 and cpn10 interact functionally with one another. However, the mechanistic details underlying the function of these ATP-dependent accessory proteins are particularly obscure.

Recently, we have used purified *Escherichia coli* chaperonins, cpn60 and cpn10, to demonstrate that the in vitro reconstitution of catalytically functional dimeric ribulosebisphosphate carboxylase (Rubisco) from an unfolded, biologically inert state depends on the presence of both chaperonins and MgATP (Goloubinoff et al., 1989b). We envision that a labile folding intermediate, Rubisco-I, is first stabilized by interaction with cpn60. Subsequently, this binary complex, cpn60–Rubisco-I, interacts with a co-chaperonin (cpn10) and MgATP in a reaction that leads to the formation of the biologically active, native Rubisco dimers. This primitive description of chaperonin function generates a host of fundamental questions. What are the structural elements that enable the chaperonins to interact with Rubisco-I but not with native Rubisco (Rubisco-N)? What happens to Rubisco subunits while they are associated with the chaperonins? By what mechanism(s) is the rate of folding enhanced? How is the hydrolysis of ATP coupled to the release of the folded protein?

Perhaps the most fundamental mechanistic question concerning any chemical reaction is its stoichiometry—in the present context, how many moles of ATP are hydrolyzed per mole of folded Rubisco? Our initial attempts to determine this stoichiometry yielded values much too large to be credible. Several complicating factors emerged. First, cpn60 alone catalyzes the hydrolysis of ATP (Ishihama et al., 1976a,b; Hendrix, 1979). While this uncoupled reaction is slow compared with those of other ATPases, it is almost 2 orders of magnitude faster than the highest rates of in vitro, chaperonin-dependent Rubisco folding observed so far. Second, cpn10 has been reported to partially inhibit this uncoupled, cpn60-dependent hydrolysis of ATP (Chandrasekhar et al., 1986).

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[1] Abbreviations: cpn10, chaperonin 10; cpn60, chaperonin 60; ribulose-P_2, ribulose 1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase; Rubisco-U, unfolded Rubisco; Rubisco-N, native Rubisco; Rubisco-I, a folding intermediate.

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It thus became apparent that, before a meaningful stoichiometry for the folding and release of Rubisco could be determined, it was necessary to understand not only the nature of the interaction between cpn60 and cpn10 but also the factors which determine whether ATP hydrolysis is coupled or uncoupled. Here, we report the results of these investigations.

Experimental Procedures

Materials. [γ-^{32}P]ATP (3400 Ci mmol^{-1}) and sodium [^{14}C]carbonate (55 Ci mol^{-1}) were from New England Nuclear. Recombinant, dimeric Rubisco was purified from *Escherichia coli* expressing a plasmid-encoded gene from *Rhodospirillum rubrum* (Pierce & Reddy, 1986; Pierce & Gutteridge, 1986). A published extinction coefficient was used to determine its concentration (Schloss et al., 1982). Ribulose-P_2 was synthesized and purified as before (Gutteridge et al., 1989).

Purification of Chaperonins. The *E. coli* chaperonins cpn60 and cpn10 were purified from lysates of cells bearing the multicopy plasmid pGroESL (Goloubinoff et al., 1989a). The purifications were modified, scaled-up versions of previously published protocols (Hendrix, 1979; Chandrasekhar et al., 1986).[2] After purification, both cpn60 and cpn10 were extensively dialyzed against 50 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol to remove unwanted monovalent cations. They were then supplemented with glycerol to a final concentration of 10% (v/v), rapidly frozen in liquid nitrogen, and stored at −80 °C.

Confirmation that the purified proteins were cpn60 and cpn10 was obtained by N-terminal sequence analyses. The protomer concentrations of cpn60 and cpn10 were measured at 280 nm with extinction coefficients determined by quantitative amino acid analyses: 3.44×10^3 M^{-1} cm^{-1} for cpn10 and 2.38×10^4 M^{-1} cm^{-1} for cpn60.

Assay of cpn60 ATPase. The release of radioactive inorganic phosphate from [γ-^{32}P]ATP was monitored as described (Lill et al., 1989) with some minor modifications. The basic reaction mixture contained 50 mM Tris-HCl, pH 7.7, 7 mM $MgCl_2$, and 0.5–5 μM cpn60 (based on protomer). Reactions were initiated by the addition of [γ-^{32}P]ATP (0.04–0.08 Ci mmol^{-1}) to concentrations specified in the figure legends. Complete reaction systems were incubated at 22 °C and were terminated at the indicated times by the removal of an aliquot (25 μL) to a microfuge tube containing 175 μL of 1 M perchloric acid and 1 mM sodium phosphate. The samples were kept on ice. Next, 0.4 mL of 20 mM ammonium molybdate and 0.4 mL of isopropyl acetate were added and the solutions vigorously mixed. The phases were separated by centrifugation (15 s at 15000*g*). One hundred microliters of the organic phase, containing the radioactive orthophosphate–molybdate complex, was removed and ^{32}P determined by liquid scintillation counting. All experiments included control reactions (lacking cpn60) to correct for the small amount of acid hydrolysis of [γ-^{32}P]ATP that occurs during the work-up.

Preparation of Unfolded Rubisco (Rubisco-U). The substrate for both spontaneous and chaperonin-dependent reconstitution experiments was prepared by denaturing native Rubisco (Rubisco-N) in guanidine hydrochloride. A small aliquot of enzyme was diluted into 6 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 7.6, and 1 mM NaEDTA (to a protomer concentration of 4–12 μM) and incubated at room temperature for at least 30 min prior to reconstitution. Exceptions to this protocol are noted in the figure legends. To quantitate the degree of reconstitution in a particular experiment, an aliquot of Rubisco-N was treated identically, except that guanidine hydrochloride was omitted.

Reconstitution of Rubisco. The protocol for chaperonin-dependent reconstitution of Rubisco was essentially as described (Goloubinoff et al., 1989b). Rubisco-U was diluted to about 68 nM and permitted to fold under the conditions described in the figure legends. Chaperonin-dependent reconstitution reactions were terminated by use of a glucose/hexokinase trap, and Rubisco activity was determined as previously described (Goloubinoff et al., 1989b).

Further experimental details are given in the figure legends.

Results

Ionic Requirements for Chaperonin 60 ATPase Activity and Rubisco Reconstitution. In the initial phase of this investigation the activities of different preparations of chaperonins were disconcertingly variable. This variation applied to both the cpn60 ATPase activity and the chaperonin-dependent reconstitution of Rubisco. Variability was eventually traced to the presence of contaminating monovalent cations, K^+ and NH_4^+, which varied from one preparation to the next. When these cations were systematically and rigorously excluded,[3] the ability of cpn60 to hydrolyze ATP and of the complete chaperonin system to reconstitute Rubisco from an unfolded state was lost. Experiments that clearly demonstrate the requirement for the monovalent cation, K^+, for both the uncoupled ATPase activity of cpn60 and the coupled release of active Rubisco are shown in Figures 1 and 2. In Figure 1A, cpn60 (alone or supplemented with a slight molar excess of cpn10) was first incubated with MgATP in the absence of K^+. In neither case was the hydrolysis of ATP observed. After 12 min potassium ion was added to 1 mM. In the absence of cpn10, the addition of K^+ initiated the uncoupled hydrolysis of ATP at a rate of about 0.1 s^{-1} (based on protomers). This rate is about a 100-fold faster than that observed in the absence of K^+. In the presence of cpn10, however, hydrolysis of ATP, even in the presence of K^+, was largely (>95%) inhibited after a brief period.

In a parallel experiment (Figure 2A) the K^+ requirement for the chaperonin-dependent reconstitution of Rubisco was demonstrated. Rubisco-U was diluted 200-fold into a solution devoid of K^+ but containing MgATP and both cpn60 and cpn10. Under these conditions no reconstitution of Rubisco occurred. However, after 12 min, when K^+ was added to 1 mM, active Rubisco was reconstituted. The brief lag period following the addition of K^+ has been attributed to the formation of catalytically active dimers from inactive, folded monomers (Goloubinoff et al., 1989b).

Since the uncoupled ATPase activity of cpn60 involves multiple catalytic turnovers, the application of standard enzyme kinetics is appropriate. By use of this approach, a value of about 80 μM K^+ was determined for $K^A_{1/2}$, the concentration of K^+ supporting a half-maximal rate (Figure 1B). In the presence of excess K^+ (50 mM), the K_m for ATP was determined to be about 7 μM. At present the in vitro reconstitution of Rubisco does not involve multiple turnovers but rather the pseudo-first-order discharge of the cpn60–Rubisco-I binary complex. This is dependent upon cpn10, MgATP, and K^+. To determine the influence of K^+ on the reconstitution of Rubisco, initial rates were measured, discounting the brief lag period described above. As indicated in Figure 2B the

[2] Copies of these protocols are available upon request to G.H.L.

[3] To avoid extraneous contaminating K^+, some caution is needed when reaction solutions are being prepared. For example, pH electrodes filled with saturated KCl should not be permitted to contact such solutions.

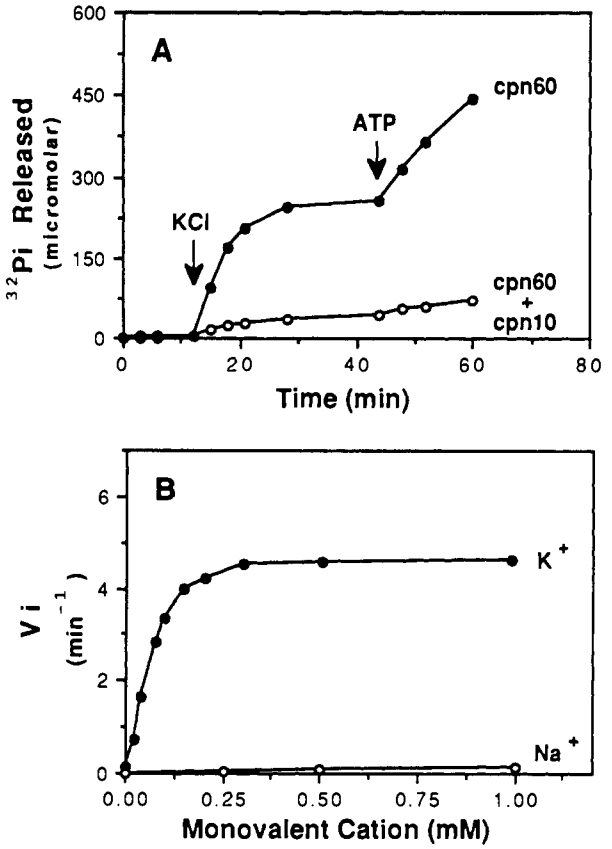


FIGURE 1: The uncoupled ATPase activity of cpn60 requires low concentrations of ionic potassium and is inhibited by cpn10. (A) cpn60 (5 μM) was incubated in the basic reaction mixture, in the presence (○) or absence (●) of cpn10 (10 μM). Reactions were initiated by addition of [γ-^{32}P]ATP to 300 μM. After 12 min, KCl was added to 1 mM, and after 44 min additional [γ-^{32}P]ATP (342 μM) was added to both reactions. At various times, reactions were terminated and assayed for radioactive P_i as described under Experimental Procedures. (B) Shown are the effects of added KCl (●) or NaCl (○) on the initial rates of [γ-^{32}P]ATP hydrolysis by cpn60. The standard reaction mixture contained 0.75 μM cpn60, supplemented with NaCl or KCl as indicated. Reactions were initiated by the addition of [γ-^{32}P]ATP to 300 μM. At appropriate times, aliquots of the reaction mixtures were analyzed as above. Initial rates (V_i) of [^{32}P]P_i release have been expressed as turnover numbers for cpn60-mediated ATP hydrolysis.

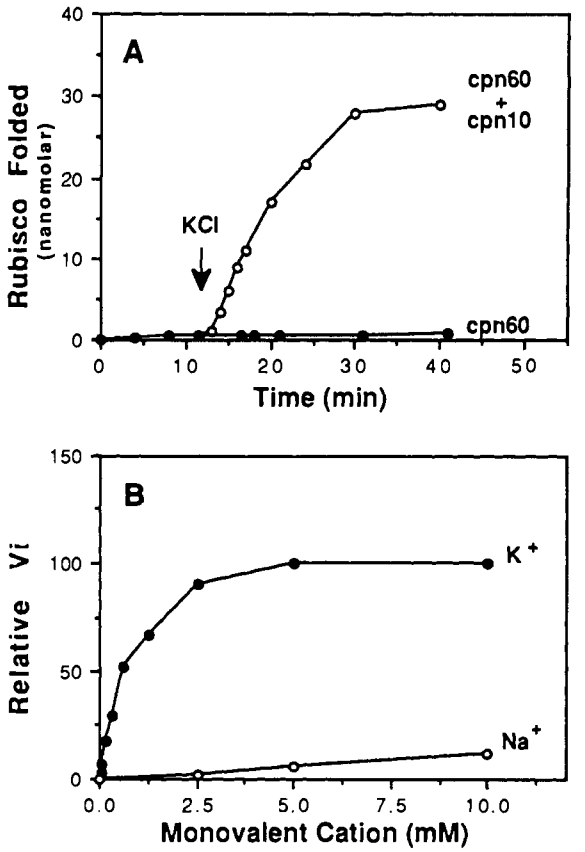


FIGURE 2: Chaperonin-dependent folding of Rubisco requires low concentrations of ionic potassium. (A) cpn60 (5 μM) was incubated at 25 °C, in the presence (○) or absence (●) of cpn10 (5 μM), in 50 mM Tris-HCl (pH 7.7), 7 mM $MgCl_2$, and 2.0 mM ATP. Reactions were initiated by the addition of Rubisco-U to 68.3 nM. After a 12-min incubation (arrow), 1 mM KCl was added. At indicated times, folding reactions were terminated and assayed for Rubisco activity as described under Experimental Procedures. (B) Shown are the effects of KCl (●) or NaCl (○) on initial rates of chaperonin-mediated Rubisco folding. Reactions were conducted at 25 °C as follows: Rubisco-U was diluted 70-fold (to 80 nM) into a solution containing 63 mM Tris-HCl (pH 7.7), 8.9 mM $MgCl_2$, and 3.13 μM cpn60. Aliquots (112 μL) of this mixture were then diluted to a final volume of 132 μL and supplemented with cpn10 (to 3.75 μM) and either KCl or NaCl, at the concentrations indicated. Control reactions received no additional monovalent cation. After all components were mixed, folding was initiated with 10 μL of ATP to 3.5 mM. At appropriate times, folding reactions were terminated and assayed for Rubisco activity as described above. Initial rates of Rubisco folding are plotted as a function of monovalent cation concentration; a relative V_i of 100 corresponds to 3.94 nM Rubisco folded per minute, the rate observed in 10 mM KCl.

reconstitution reaction was also saturated by rather low concentrations of K^+. A value of about 0.6 mM K^+ was determined for $K^F_{1/2}$, the concentration of K^+ supporting a half-maximal rate of reconstitution.

The ability of other monovalent cations to support both the uncoupled ATPase activity of cpn60 and the chaperonin-dependent reconstitution of Rubisco was explored (Figure 3). Initial rates were measured with 1 mM monovalent cation for the ATPase activity and 14 mM for Rubisco reconstitution, concentrations that are saturating for K^+. In common with other K^+-requiring enzymes (Evans & Sorger, 1966; Suelter, 1974), both of these cpn60-dependent reactions were substantially activated by Rb^+ and NH_4^+. The other monovalent cations, Na^+, Li^+, and Cs^+, were virtually ineffective.

Spontaneous Reconstitution of Rubisco Is Strongly Temperature Dependent. Previous attempts to reconstitute Rubisco spontaneously were conducted at 25 °C for periods up to 2 h (Goloubinoff et al., 1989b). Under those conditions very little (<2%) reconstitution was observed in the absence of the chaperonin system. Recently, we have found that Rubisco-U can fold spontaneously but displays a rather sharp temperature dependency (Figure 4). Thus, at temperatures above 30 °C no spontaneous reconstitution could be detected.[4] Consistent with our previous results, only a small amount (<10%) of active Rubisco could be detected after a 24-h incubation at 25 °C, and this did not increase with further incubation. However, at temperatures below 25 °C the maximum yield of spontaneously reconstituted enzyme rose sharply (Figure 4), such that all of the activity could be recovered at temperatures below about 10 °C.

Chaperonin-Dependent Refolding of Rubisco Is Faster Than the Spontaneous Reaction; Chaperonins Inhibit the Spontaneous Reaction. Although Rubisco folded spontaneously at 15 °C, chaperonins still conferred about a 10-fold increase on the rate of reconstitution (Figure 5). In contrast to the chaperonin-dependent reaction, spontaneous reconstitution occurred whether or not K^+ was present. Furthermore,

[4] Chaperonin-facilitated reconstitution of Rubisco could still be observed up to temperatures of at least 37 °C.

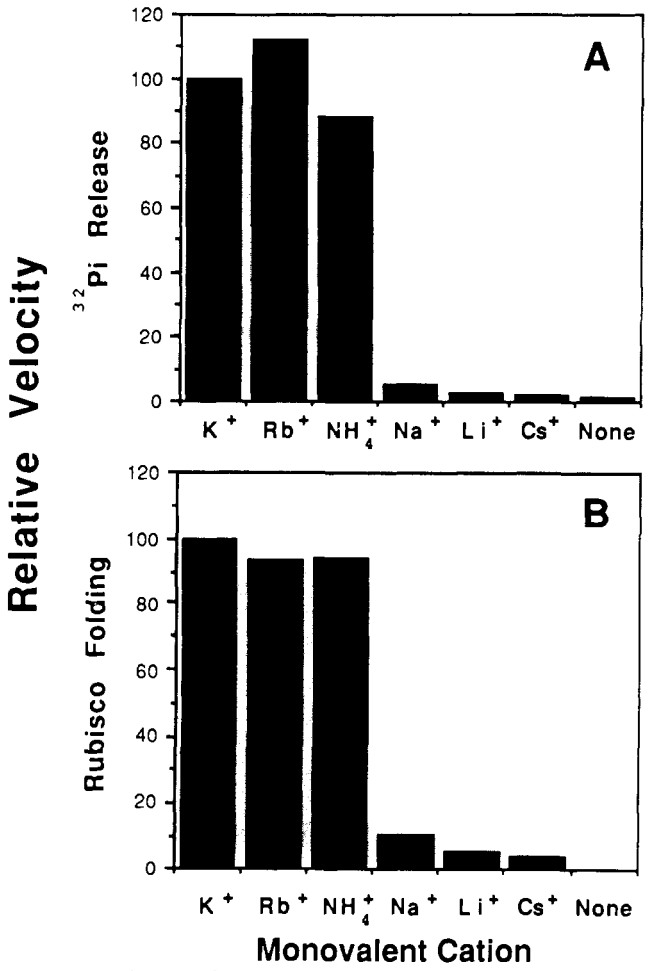


FIGURE 3: Influence of monovalent cations on the uncoupled ATPase activity of cpn60 and chaperonin-dependent folding of Rubisco. (A) ATPase assays were conducted in the basic reaction mixture, supplemented with 1.0 mM monovalent cation, added as chloride salts. Reactions also contained 1.5 μM cpn60 and were initiated by the addition of [γ-^{32}P]ATP to 300 μM. At appropriate times, reactions were terminated and assayed for [^{32}P]P_i release as described under Experimental Procedures. Initial rates have been expressed relative to the turnover number in 1.0 mM KCl (6.0 min^{-1}). (B) Folding reactions were constructed as described in Figure 2B with the following exceptions: monovalent cations (added as chloride salts) and ATP were present at final concentrations of 14 and 1.82 mM, respectively. At appropriate times, folding reactions were terminated and assayed for Rubisco activity as described under Experimental Procedures. Initial rates of Rubisco folding have been expressed relative to the rate in 14 mM KCl (2.64 nM Rubisco folded per minute).

spontaneous reconstitution was inhibited by the presence of a molar excess of cpn60 over Rubisco. This result was not unexpected since we previously reported that cpn60 reacts with an unstable folding intermediate, Rubisco-I, to form a stable binary complex of cpn60–Rubisco-I (Goloubinoff et al., 1989b). After a 16-h incubation, when K^+ and ATP were added to complete the reaction mixture, active Rubisco could still be discharged (Figure 5), further demonstrating the stability of this complex.

Conditions for Forming the Binary cpn60–cpn10 Complex. Inhibition of the uncoupled ATPase activity of cpn60 by cpn10 implies the formation of a binary complex between the two. Such a complex has been reported to occur in the presence of MgATP (Chandrasekhar et al., 1986). In that study the cpn60–cpn10 complex did not form in the presence of non-hydrolyzable analogues of ATP, suggesting that hydrolysis was necessary. In the present study, cpn10 inhibited the uncoupled ATPase activity of cpn60 to a much greater degree than that previously reported. However, inhibition was not instantaneous

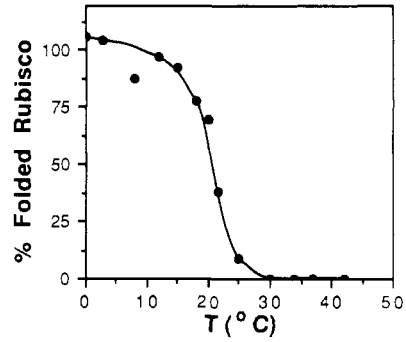


FIGURE 4: Spontaneous folding of Rubisco-U as a function of temperature. Five microliters of 13.7 μM Rubisco-U in 4.8 M guanidine hydrochloride, 80 mM Tris-HCl, pH 7.7, 0.8 mM EDTA, and 0.1 M dithiothreitol were diluted into 995 μL of a solution containing 0.1 M Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM KCl, and 5 μM bovine serum albumin at the indicated temperatures. Controls containing an equal quantity of Rubisco-N were also prepared. The solutions were incubated at the indicated temperatures. After 5, 19, 24, and 44 h, 200-μL aliquots were withdrawn, brought to 25 °C, and assayed for Rubisco activity as described under Experimental Procedures. The data points show the extent of spontaneous folding after 44 h. However, these values were not significantly different from those observed after 24 h, indicating that the reactions were essentially complete by 24 h at all temperatures.

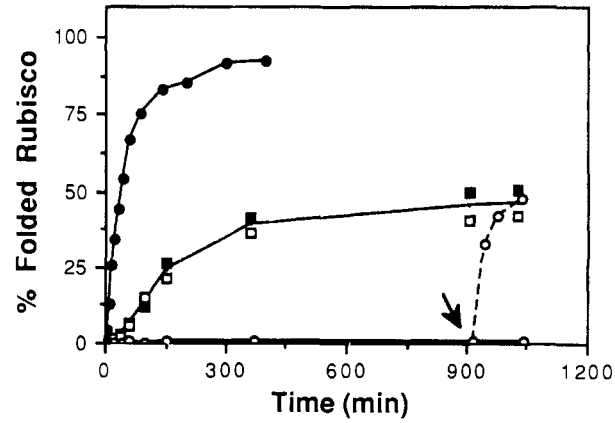


FIGURE 5: Comparison of the spontaneous and the chaperonin-dependent folding of Rubisco at 15 °C. Spontaneous folding of Rubisco in the presence (■) and absence (□) of K^+. The folding of Rubisco at 15 °C was enhanced by the complete chaperonin system (●) but inhibited when that system lacks K^+ and ATP (○). When these missing components were added after 15 h, active Rubisco was formed (broken line). Rubisco-U was prepared as in the legend to Figure 4. For the spontaneous reactions 15 μL of 13.7 μM Rubisco-U was diluted into 2985 μL of a solution of 50 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, and 5 μM bovine serum albumin containing either no KCl (□) or 10 mM KCl (■) at 15 °C. Controls containing an equal quantity of Rubisco-N were prepared. Aliquots were withdrawn at the times indicated, and Rubisco activity was determined at 15 °C as described under Experimental Procedures. For the complete chaperonin-dependent reaction (●) 15 μL of 13.7 μM Rubisco-U was diluted into 2985 μL of a solution of 50 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 10 mM KCl, 5 μM bovine serum albumin, 5.1 μM cpn60, 5.3 μM cpn10, and 2 mM ATP at 15 °C. A control containing an equal quantity of Rubisco-N was prepared to establish 100% reconstitution. For the incomplete chaperonin reaction (○) ATP and KCl were omitted from the reaction solution. These were added after 915 min (arrow) to give final concentrations of 2 mM ATP and 10 mM KCl. Aliquots were withdrawn at the indicated times and quenched with glucose/hexokinase, and Rubisco activity was determined at 15 °C as described under Experimental Procedures.

but required several minutes to develop, during which time cpn60 catalyzed several rounds of ATP hydrolysis. In light of these observations it was of interest to determine whether K^+ was necessary for the formation of the cpn60–cpn10 complex. To address this question, a series of gel filtration experiments were performed (Figure 6).

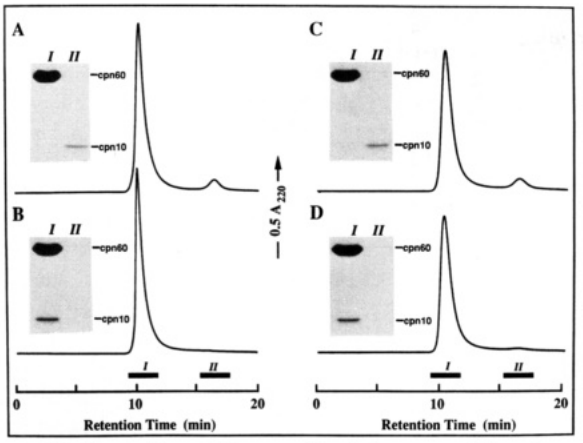


FIGURE 6: Formation of the complex between cpn60 and cpn10 requires the presence of ATP but does not require ionic potassium. The basic reaction mixture contained 70 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 12.2 μM cpn60, and 7.2 μM cpn10. In addition, the reactions shown in Panels B and D contained 0.6 mM ATP while those shown in Panels A and B also contained 10 mM KCl. Reactions were conducted at 22 °C and were initiated by the addition of cpn60. After a 12-min incubation period, aliquots (200 μL) of the reaction mixtures were injected onto an HPLC-TSK sizing column (type G3000SW, 7.5 × 600 mm). The basic column buffer contained 100 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$. In addition, the buffers used for Panels B and D contained 0.25 mM ATP while those used for Panels A and B contained 10 mM KCl. Proteins were eluted at room temperature at a flow rate of 1.0 mL min^{-1}, and the absorption at 220 nm was monitored. As indicated, column fractions corresponding to the positions of cpn60 (peak I, 9.5–11.5 min) and cpn10 (peak II, 15.5–17.5 min) were collected and subjected to SDS–PAGE, after precipitation of the proteins with 80% acetone. Note that recovery of cpn10 in peak II is only about 50% during the acetone precipitation step.

Mixtures containing a slight molar excess of cpn60 over cpn10 were equilibrated with and without MgATP (600 μM) in the presence (10 mM) or absence of K^+. The four possible reactions were then applied to gel filtration columns, equilibrated with the appropriate combination of nucleotide and K^+. As anticipated, a complex between cpn60 and cpn10 was not observed in the absence of ATP, regardless of the presence or absence of K^+ (Figure 6A,C). Under such conditions, the column eluate emerging between 9.5 and 11.5 min (peak I) contained only cpn60, while that emerging between 15.5 and 17.5 min (peak II) contained only cpn10. In contrast, when both ATP and K^+ were present (Figure 6B), only a single peak was detected at 10.5 min. That this peak contained the cpn60–cpn10 complex was revealed by SDS–PAGE of the eluate corresponding to peak I, which now contained both chaperonins. No detectable cpn10 was present in the eluate emerging between 15.5 and 17.5 min. Next, the experiment was repeated in the presence of ATP, but in the absence of K^+ (Figure 6D).[5] This revealed that K^+ was not necessary for formation and recovery of the cpn60–cpn10 complex. These results suggest that continued ATP hydrolysis is not necessary for complex formation. However, we cannot exclude the possibility that a single turnover of ATP occurred.

Discussion

We have demonstrated here that both the chaperonin- and MgATP-dependent reconstitution of unfolded Rubisco and the uncoupled ATPase activity of cpn60 require ionic potassium. These observations provide important clues regarding the mechanism by which cpn60 hydrolyzes ATP. Indeed, it is known that K^+ plays an important role for a number of other proteins which cleave ATP. As with other K^+-requiring systems, NH_4^+ and Rb^+ substitute most effectively (Suelter, 1974), while Na^+, Li^+, and Cs^+ are only partly effective at higher concentrations. Furthermore, the potassium activation constants for the uncoupled ATPase activity of cpn60 and the chaperonin-dependent reconstitution of Rubisco (0.08 and 0.6 mM, respectively) are among the lowest recorded. Other are typically an order of magnitude larger (Suelter, 1974). The precise role of K^+ is presently unknown; however, it is likely that a common site for K^+ on cpn60 is responsible for both the coupled and uncoupled hydrolysis of ATP.

In contrast, K^+ is unnecessary for the spontaneous folding of Rubisco observed at lower temperatures (Figures 4 and 5). It would appear that the principal requirement for spontaneous folding is to minimize the formation of biologically unproductive aggregates. Presumably, intermolecular aggregation is suppressed at lower temperatures, enabling proper intramolecular folding reactions to predominate. This conclusion is supported by the sharp decline in the final yield of spontaneously folded Rubisco as the temperature is increased (Figure 4). At higher temperatures, restrictive for spontaneous folding, the formation of aggregates is largely avoided by a stable interaction of Rubisco-I with the $[cpn60]_{14}$ "double donut". This interaction also occurs at lower temperatures. Thus, at 15 °C the spontaneous folding of Rubisco can be arrested by the presence of a molar excess of cpn60 (Figure 5). This interaction must be rather stable considering that, in the presence of cpn60, no spontaneous folding was observed over a 15-h period. Nevertheless, folded Rubisco could still be recovered at this time by addition of the missing components of the reaction, K^+ and MgATP (Figure 5).

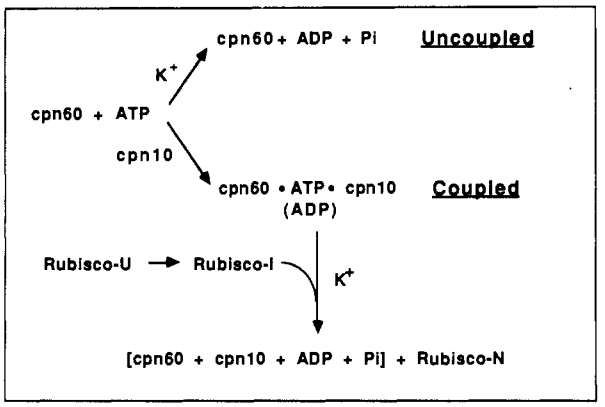
At 15 °C, the chaperonin-mediated rate enhancement of reconstitution could be assessed since sufficient Rubisco folds spontaneously for a reliable rate measurement to be made. In vitro, this rate enhancement is about 10-fold, although it may be larger in vivo. Although the rate enhancement is not large, it is perhaps what one might expect if Rubisco, while bound to cpn60, is restrained from exploring biologically unproductive folding pathways. Regardless of the origin of the rate enhancement, the fact that Rubisco folds spontaneously establishes that chaperonins intervene at a kinetic rather than a thermodynamic level.

Cpn60 catalyzes the hydrolysis of ATP with a k_{cat} of about 0.1 s^{-1} (based on protomer). This is rather slow compared with other ATPases. For example, dog kidney ($Na^+ + K^+$)-ATPase turns over at about 33 s^{-1} (Miller & Farley, 1990). The "weak" ATPase activity of cpn60 probably represents an uncoupled artifact of the in vitro system, in the same way that hexokinase hydrolyzes ATP in the absence of glucose. There is no obvious reason why cpn60 should catalyze the wasteful hydrolysis of ATP in vivo. Nevertheless, the K^+- and Mg^{2+}-dependent ATPase activity of cpn60 demonstrates that this chaperonin binds K^+, Mg^{2+}, and ATP.

The fact that K^+ is needed for ATP hydrolysis and Rubisco reconstitution suggests a novel role for cpn10. Consider the following observations: (1) cpn10 is needed to bring about the MgATP-dependent discharge of the cpn60–Rubisco-I binary complex, which leads to the formation of active Rubisco; (2) cpn10 virtually abolishes the uncoupled hydrolysis of ATP by cpn60; (3) cpn10, K^+, and MgATP are necessary for Rubisco reconstitution. These observations raise the possibility that an important function of cpn10 is to serve as a "coupling factor", to couple the K^+-dependent hydrolysis of ATP to the

[5] Recent experiments employing a new TSK column failed to show the broadening of peak I that was apparent in the absence of K^+ (traces C and D of Figure 6). The results with the new column are otherwise identical with those shown.

Scheme I: Cpn10 Couples cpn60-Dependent ATP Hydrolysis to the Folding and/or Release of a Target Polypeptide[a]



[a] In the absence of the co-chaperonin cpn10, hydrolysis of ATP by cpn60 is "uncoupled" and requires ionic potassium. In the presence of ATP, cpn10 forms a complex with cpn60, regardless of the presence or absence of K^+. The fact that this complex hydrolyzes ATP poorly (even in the presence of K^+) suggests that cpn10 may be a "coupling factor". Thus, significant hydrolysis of ATP by the cpn10–cpn60 complex should occur only in the presence of K^+ and an unfolded or partly folded protein, such as Rubisco-I. For additional details see text.

release of the folded target protein from cpn60. This idea does not preclude additional roles for cpn10, such as the displacement of the folded or partially folded protein from cpn60.

As shown in Scheme I, cpn10 profoundly and reciprocally alters the two activities associated with cpn60, i.e., the uncoupled hydrolysis of ATP and the discharge of bound Rubisco. (1) In the absence of cpn10, the hydrolysis of ATP is uncoupled. (2) When cpn10 is present, a complex is created that is hardly capable of hydrolyzing ATP, at least not in the absence of an unfolded or partly folded polypeptide (Figure 1A). In this respect cpn10 acts to couple the hydrolysis of ATP to biologically productive work, the folding and/or the release of the target protein from cpn60. This characteristic, a properly coupled hydrolysis of ATP, is shared by members of the hsp70 family. Flynn et al. (1989) have shown that the binding of peptides to hsc70 and BiP promotes the hydrolysis of ATP that is coupled to the release of the bound peptide. Apparently this family of molecular chaperones does not require a co-chaperone, such as cpn10, to perform the coupling role. The mechanism by which cpn10 couples the K^+-dependent hydrolysis of ATP to the release of the folded protein is not understood at present.

The fundamental question of the stoichiometry of ATP hydrolysis associated with the chaperonin-dependent folding of Rubisco remains to be addressed. Nevertheless, experimental difficulties remain to be overcome before meaningful measurements can be made. Presently, we are reconstituting picomolar quantities of Rubisco in the presence of micromolar quantities of ATP. Despite being able to largely control the uncoupled hydrolysis of ATP, a determination of stoichiometry, under these conditions, is likely to be accompanied by a large experimental error. Accordingly, our next goal is to amplify the quantity of Rubisco that is reconstituted by the chaperonins. To do so requires that the chaperonins function catalytically (i.e., undergo more than one turnover). This requires finding conditions under which the folding intermediate, Rubisco-I, is sufficiently stable to await its conversion to the native state by catalytic quantities of chaperonins, without undergoing aggregation. A detailed accounting of the spontaneous folding of Rubisco may reveal such conditions.

The ability of Rubisco-U to fold spontaneously at lower temperatures (Figure 4) clearly demonstrates that the energy released upon hydrolysis of ATP by the chaperonin system is not conserved in the folded protein. Spontaneous folding would not otherwise occur. How then might the chaperonins function? One of the major problems to be overcome in any protein folding experiment is the propensity of the unfolded protein to form biologically unproductive aggregates. Rubisco is no exception in this regard. From the results reported here and previously (Barraclough & Ellis, 1980; Roy et al., 1983; Bochkareva et al., 1988; Ostermann et al., 1989; Goloubinoff et al., 1989b; Lubben et al., 1989), it is clear that the oligomer $[cpn60]_{14}$ can form rather stable binary complexes with a wide variety of unfolded or partly folded proteins in vitro, in vivo, and in organello. By sequestering nascent polypeptides in the form of a stable binary complex, chaperonins suppress interactions that would otherwise lead to aggregation. Folding of these proteins can then occur while they are immobilized on the chaperonin. The folding of several proteins while attached to insoluble matrices has been reported (Epstein & Anfinsen, 1962; Sinha & Light, 1975; Janolino et al., 1978; Creighton, 1986; Hoess et al., 1988). We propose that cpn60 is the biological equivalent of these insoluble matrices, permitting unfolded polypeptides to undergo folding rather than aggregation. The structural elements that enable cpn60 to bind unfolded proteins but not native proteins remain to be defined. The binding energy associated with formation of the binary complex may also preclude the dissociation of the folded protein. This step requires the presence of the "coupling factor" (cpn10) and K^+ and is associated with the hydrolysis of ATP. We suggest that the free energy associated with ATP hydrolysis is harnessed to overcome the binding forces holding the target protein to the chaperonin.

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References

Barraclough, R., & Ellis, R. J. (1980) *Biochim. Biophys. Acta 608*, 19–31.

Bochkareva, E. S., Lissin, N. M., & Girshovich, A. S. (1988) *Nature 336*, 254–257.

Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R., & Georgeopoulos, C. (1986) *J. Biol. Chem. 261*, 12414–12419.

Creighton, T. E. (1985) in *Protein Structure, Folding and Design* (Oxender, D. L., Ed.) pp 249–257, Alan R. Liss, New York.

Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends Biochem. Sci. 14*, 339–342.

Epstein, C. J., & Anfinsen, C. B. (1962) *J. Biol. Chem. 237*, 2175–2179.

Evans, H. J., & Sorger, G. J. (1966) *Annu. Rev. Plant Physiol. 17*, 47–76.

Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29*, 2205–2212.

Fischer, G., Bang, H., & Mech, C. (1984) *Biomed. Biochim. Acta 43*, 1101–1111.

Flynn, G. C., Chappell, T. G., & Rothman, J. E. (1989) *Science 245*, 385–390.

Freedman, R. B. (1989) *Cell 57*, 1069–1072.

Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989a) *Nature 337*, 44–47.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A., & Lorimer, G. H. (1989b) *Nature 342*, 884–889.

Gutteridge, S., Reddy, G. S., & Lorimer, G. H. (1989) *Biochem. J. 260*, 711-716.
Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgeopoulos, C., Hendrix, R. W., & Ellis, R. J. (1988) *Nature 333*, 330-334.
Hendrix, R. W. (1979) *J. Mol. Biol. 129*, 375-392.
Hoess, A., Arthur, A. K., Wanner, G., & Fanning, E. (1988) *Bio/Technology 6*, 1214-1217.
Hohn, T., Hohn, B., Engel, A., Wurtz, M., & Smith, P. R. (1979) *J. Mol. Biol. 129*, 359-373.
Ishihama, A., Ikeuchi, T., & Yura, T. (1976a) *J. Biochem. (Tokyo) 79*, 917-925.
Ishihama, A., Ikeuchi, T., Matsumoto, A., & Yamamoto, S. (1976b) *J. Biochem. (Tokyo) 79*, 927-936.
Janolino, V. G., Sliwkowski, M. X., Swaisgood, H. E., & Horton, H. R. (1978) *Arch. Biochem. Biophys. 191*, 269-277.
Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y., & Ito, K. (1989) *EMBO J. 8*, 3517-3522.
Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D., & Wickner, W. (1989) *EMBO J. 8*, 961-966.
Lubben, T. H., Donaldson, G. K., Viitanen, P. V., & Gatenby, A. A. (1989) *Plant Cell 1*, 1223-1230.
McMullin, T. W., & Hallberg, R. L. (1988) *Mol. Cell. Biol. 8*, 371-380.
Miller, R. P., & Farley, R. A. (1990) *Biochemistry 29*, 1524-1532.
Ostermann, J., Horwich, A. L., Neupert, W., & Hartl, F.-U. (1989) *Nature 341*, 125-130.
Picketts, D. J., Mayanil, C. S. K., & Gupta, R. S. (1989) *J. Biol. Chem. 264*, 12001-12008.
Pierce, J., & Gutteridge, S. (1986) *Appl. Environ. Microbiol. 49*, 1094-1100.
Pierce, J., & Reddy, G. S. (1986) *Arch. Biochem. Biophys. 245*, 483-493.
Roy, H., Bloom, M., Milos, P., & Monroe, M. (1982) *J. Cell Biol. 94*, 20-27.
Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D., & Harman, F. C. (1982) *Methods Enzymol. 90*, 522-528.
Sinha, N. K., & Light, A. (1975) *J. Biol. Chem. 250*, 8624-8629.
Suelter, C. H. (1974) *Met. Ions Biol. Syst. 3*, 201-251.
Tilly, K., & Georgeopoulos, C. P. (1982) *J. Bacteriol. 149*, 1082-1088.
Van Dyk, T. K., Gatenby, A. A., & LaRossa, R. A. (1989) *Nature 342*, 451-453.
Zweig, M., & Cummings, D. J. (1973) *J. Mol. Biol. 80*, 505-518.

Identification and Localization of Bound Internal Water in the Solution Structure of Interleukin 1β by Heteronuclear Three-Dimensional ^{1}H Rotating-Frame Overhauser ^{15}N-^{1}H Multiple Quantum Coherence NMR Spectroscopy†

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ABSTRACT: The presence and location of bound internal water molecules in the solution structure of interleukin 1β have been investigated by means of three-dimensional ^{1}H rotating-frame Overhauser ^{1}H-^{15}N multiple quantum coherence spectroscopy (ROESY-HMQC). In this experiment through-space rotating-frame Overhauser (ROE) interactions between NH protons and bound water separated by $\leq$3.5 Å are clearly distinguished from chemical exchange effects, as the cross-peaks for these two processes are of opposite sign. The identification of ROEs between NH protons and water is rendered simple by spreading out the spectrum into a third dimension according to the ^{15}N chemical shift of the directly bonded nitrogen atoms. By this means, the problems that prevent, in all but a very few limited cases, the interpretation, identification, and assignment of ROE peaks between NH protons and water in a 2D ^{1}H-^{1}H ROESY spectrum of a large protein such as interleukin 1β, namely, extensive NH chemical shift degeneracy and ROE peaks obscured by much stronger chemical exchange peaks, are completely circumvented. We demonstrate the existence of 15 NH protons that are close to bound water molecules. From an examination of the crystal structure of interleukin 1β [Finzel, B. C., Clancy, L. L., Holland, D. R., Muchmore, S. W., Watenpaugh, K. D., & Einspahr, H. M. (1989) *J. Mol. Biol. 209*, 779-791], the results can be attributed to 11 water molecules that are involved in interactions bridging hydrogen-bonding interactions with backbone amide and carbonyl groups which stabilize the 3-fold pseudosymmetric topology of interleukin 1β and thus constitute an integral part of the protein structure in solution.

Bound water molecules are invariably present in high-resolution X-ray structures where they may be found either within the interior of the protein or on the surface (Deisenhofer & Steigemann, 1975; Blundell & James, 1976; Finney, 1979; James & Sielecki, 1983; Edsall & McKenzie, 1983; Baker & Hubbard, 1984; Teeter, 1984; Wlodawer et al., 1987). Internal water molecules often play a role in stabilizing the protein structure through bridging hydrogen-bonding interactions and additionally may be involved in catalysis (Bode & Schwager,

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