Conformational States of Ribulosebisphosphate Carboxylase and Their Interaction with Chaperonin 60

Saskia M. van der Vies,[‡] Paul V. Viitanen,[‡] Anthony A. Gatenby,[‡] George H. Lorimer,*[,‡] and Rainer Jaenicke[§]

Molecular Biology Division, Central Research and Development Department, Dupont Company Experimental Station, Wilmington, Delaware 19880-0402, and Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Universitätsstrasse 31, D-8400 Regensburg, Germany

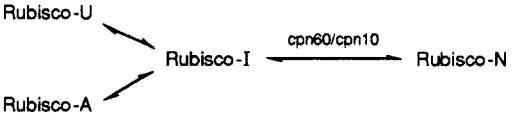
Received November 13, 1991; Revised Manuscript Received January 31, 1992

ABSTRACT: Conformational states of ribulosebisphosphate carboxylase (Rubisco) from *Rhodospirillum rubrum* were examined by far-UV circular dichroism (CD), tryptophan fluorescence, and 1-anilinonaphthalenesulfonate (ANS) binding. At pH 2 and low ionic strength ($I = 0.01$), Rubisco adopts an unfolded, monomeric conformation (UA_1 state) as judged by far-UV CD and tryptophan fluorescence. As with other acid-unfolded proteins [Goto, Y., Calciano, L. J., & Fink, A. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577], an intermediate conformation (A_1 state) is observed at pH 2 and high ionic strength. The A_1 state has an α-helical content equivalent to 64% of that present in the native dimer (N_2 state). However, fluorescence measurements indicate that the tertiary structure of the A_1 state is largely disordered. A site-directed mutant, K168E, which exists as a stable monomer [Mural, R. J., Soper, T. S., Larimer, F. W., & Hartman, F. C. (1990) *J. Biol. Chem. 265*, 6501–6505] was used to characterize the "native" monomer (N_1 state). The far-UV CD spectra of the N_1 and N_2 states are almost identical, indicating a similar secondary structure content. However, the tertiary structure of the N_1 state is less ordered than that of the N_2 state. Nevertheless, when appropriately complemented in vitro, K168E forms an active heterodimer. Upon neutralization of acid-denatured Rubisco or dilution of guanidine hydrochloride-denatured Rubisco, unstable folding intermediates (I_1 state) are rapidly formed. At concentrations at or below the "critical aggregation concentration" (CAC), the I_1 state reverts spontaneously but slowly to the native states with high yield (>65%). The CAC is temperature-dependent. At concentrations above the CAC, the I_1 and the A_1 states undergo irreversible aggregation. The commitment to aggregation is rapid [cf. Goldberg, M. E., Rudolph, R., & Jaenicke, R. (1991) *Biochemistry 30*, 2790–2797] and proceeds until the concentration of folding intermediate(s) has fallen to the CAC. In the presence of a molar excess of chaperonin 60 oligomers, the I_1 state forms a stable binary complex. No stable binary complex between chaperonin 60 and the N_1 state could be detected. Formation of the chaperonin 60–I_1 binary complex arrests the spontaneous folding process. The I_1 state becomes resistant to interaction with chaperonin 60 with kinetics indistinguishable from those associated with the appearance of the native states. In vitro complementation analysis indicated that the product of the chaperonin-facilitated process is monomeric. Spectral analyses of the I_1 state, performed at concentrations below the CAC and before significant reversal to the native states had occurred, show that it possesses a similar secondary structure content to the A_1 state but, like the N_1 state, lacks the organized tertiary structure typical of the N_2 state. The I_1 state is considerably more sensitive to proteolysis than the N_2 state, whether free in solution or bound to chaperonin 60.

The in vivo folding of some proteins does not proceed spontaneously as was once thought, but may instead be facilitated by a group of proteins, collectively referred to as chaperonins (Ellis & van der Vies, 1991). Evidence in support of this view was recently obtained with the development of an in vitro system, employing the chaperonin proteins chaperonin 60 (cpn60)[1] (groEL) and cpn10 (groES), purified from *Escherichia coli*, to facilitate the refolding of unfolded Rubisco (Goloubinoff et al., 1989a; Viitanen et al., 1990). Cpn-facilitated folding of several other proteins has also been documented (Laminet et al., 1990; Buchner et al., 1991; Badcoe et al., 1991; Mendoza et al., 1991; Martin et al., 1991; Viitanen et al., 1991; Taguchi et al., 1991).

The substrate for the Rubisco refolding experiments was either Rubisco-U, prepared by completely unfolding the native enzyme with the chaotropes urea or guanidine hydrochloride, or Rubisco-A, prepared by partly unfolding the native state with acid. Although Rubisco-U and Rubisco-A are structurally quite different, as judged by CD spectroscopy, their chaperonin-dependent conversion to the native state could be described by the same first-order rate constant. This suggested that the refolding of Rubisco-U and Rubisco-A involved a common folding intermediate, Rubisco-I. Formation of Rubisco-I was assumed to occur independently of the chaperonins. However, its conversion to the native state was rate-determining and, in some manner, cpn-dependent (Scheme I).

Scheme I



* Address correspondence to this author.
‡ Dupont Company Experimental Station.
§ Universität Regensburg.

[1] Abbreviations: cpn60, chaperonin 60; $[cpn60]_{14}$, native oligomeric form of cpn60; cpn10, chaperonin 10; Gdn·HCl, guanidine hydrochloride; Rubisco, ribulose-1,5-bisphosphate carboxylase; ANS, 1-anilinonaphthalene-8-sulfonic acid; CAC, critical aggregation concentration.

0006-2960/92/0431-3635$03.00/0

The chaperonin-facilitated refolding of Rubisco can be dissected into two discrete steps. In the first step, a nonnative state of the protein, Rubisco-I, is stabilized by forming a binary complex with cpn60:

$$\text{Rubisco-I} + \text{cpn60} \rightleftharpoons \text{cpn60·Rubisco-I}$$

In the second step, initiated by the addition of cpn10 and MgATP, Rubisco is released and reverts to the native state. The extent to which conversion to the native state occurs on the cpn60 is presently unknown:

$$\text{cpn60·Rubisco-I} \xrightarrow{[\text{cpn10, MgATP, K}^+]} \text{cpn60} + \text{Rubisco-N}$$

Our working hypothesis envisages that cpn 60 interacts with intermediate(s) in the folding process rather than with the completely unfolded state. It is based in part upon the mounting evidence [reviewed by Kuwajima (1989), Creighton (1990), and Jaenicke (1991)] that the formation of secondary structure occurs on a millisecond time scale. Thus, the lifetime of a completely unfolded protein after reestablishment of folding conditions is likely to be very short indeed. Instead, the protein rapidly forms what has been referred to as the "compact intermediate" or "molten globule" state (Ptitsyn, 1987; Kuwajima, 1989; Kim & Baldwin, 1990). Here we describe experiments to test this model and report that cpn60 interacts with a monomeric, nonnative state of Rubisco with spectral properties indicative of folding intermediate(s).

Experimental Procedures

Materials. 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 ($\epsilon_{280nm} = 6.72 \times 10^4$ M^{-1} cm^{-1}) was used to determine its concentration (Schloss et al., 1982). The plasmid pFL174, expressing the gene for the site-directed monomeric mutant Rubisco K168E,[2] was the gift of Drs. F. W. Larimer and F. C. Hartman and was grown in *E. coli* as previously described (Niyogi et al., 1986; Mural et al., 1990). The site-directed dimeric mutant Rubisco N111V was provided as purified protein by Dr. S. Gutteridge. The *E. coli* chaperonins cpn60 and cpn10 were purified from lysates of cells bearing the multicopy plasmid pGroESL (Goloubinoff et al., 1989b). The purification protocols (available on request from G.H.L.) were modified, scaled-up versions of previously published methods (Hendrix, 1979; Chandrasekhar et al., 1986). The protomer concentrations of cpn60 and cpn10 were determined by quantitative amino acid analyses with reference to the published (deduced) amino acid sequence (Hemmingsen et al., 1988). Ribulose 1,5 bisphosphate was prepared as before (Gutteridge et al., 1989). All radiochemicals were supplied by New England Nuclear.

Purification of Monomeric Mutant Rubisco K168E. Because K168E was much more susceptible to degradation by adventitious proteases during purification, modifications to the previously described protocol (Lorimer & Hartman, 1988) were necessary. The buffer in which the cells were lysed was supplemented with 0.5 μg/mL leupeptin and 0.68 μg/mL pepstatin. Immediately following chromatography, glycerol to 10% (v/v) was added to the fractions which were frozen at −80 °C until the next purification step was undertaken. Following ion-exchange chromatography as before (Lorimer & Hartman, 1988), the fractions containing K168E were pooled, concentrated, and applied to a Pharmacia TSK sizing column (type G3000SW, 7.5 × 600 mm) equilibrated with 0.1 M Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM dithiothreitol. The fractions containing K168E were pooled, and the buffer was exchanged for 50 mM potassium phosphate, pH 7.1, containing 1.7 M $(NH_4)_2SO_4$ (buffer A). The sample was applied to a Pharmacia phenyl-Superose 10/10 column equilibrated with buffer A and eluted with a 175-mL linear gradient [1.7–0 M $(NH_4)_2SO_4$] in 50 mM potassium phosphate, pH 7.1. The fractions containing K168E were pooled and concentrated, and the buffer was exchanged for 2 mM Tris-HCl, pH 7.6, and 1 mM dithiothreitol. Glycerol was added to 10% (v/v) and the purified K168E protein stored at −80 °C. Since K168E is devoid of enzymatic activity, it was detected by SDS–PAGE with the Phastgel system (Pharmacia LKB Biotechnology) and by Western blot analysis (Olsson & Olsson, 1986).

Preparation of the Various Conformational States of Rubisco. Six conformational states of Rubisco were investigated, two unfolded states, UA_1 and UG_1, which correspond to low ionic strength, acid-denatured protein and Gdn·HCl-denatured protein, respectively; two partly folded states, A_1 and I_1, which correspond to high ionic strength, acid-denatured protein and a folding intermediate, respectively; and two folded states, N_1 and N_2, which correspond to the native monomer and dimer, respectively. The UA_1 state was prepared by diluting Rubisco-N_2 in 2 mM Tris-HCl, pH 7.6, with an equal volume of 20 mM HCl. The UG_1 state was prepared by diluting a small volume of Rubisco-N_2 into 6 M Gdn·HCl, 0.1 M Tris-HCl, pH 7.6, 0.1 M dithiothreitol, and 1 mM EDTA. The A_1 state was prepared in a similar manner to the UA_1 state, except that the final solution contained, in addition, 50 mM Na_2SO_4. The I_1 state was prepared by diluting either Rubisco-UA_1 or Rubisco-UG_1 into a buffer at neutral pH. The N_1 state was examined as the stable monomeric mutant K168E.

Ultracentrifugation. Sedimentation experiments were performed on solutions of Rubisco-N_2 and Rubisco-UA_1, prepared as described above, in a Beckman Spinco Model E analytical ultracentrifuge equipped with a high-intensity light source and a UV scanning system. Double-sector cells (12-mm path) with sapphire windows were used in an AnF-Ti rotor. To detect possible concentration-dependent dissociation, meniscus depletion runs were performed at 20 900 and 10 000 rpm (Yphantis, 1964); scanning wavelengths were 230 and 280 nm, respectively. s values were determined at 44 000 rpm, plotting log r versus time and correcting for 20 °C and water viscosity. Sedimentation equilibria were evaluated from ln c versus r^2 plots, making use of a computer program developed by G. Bohm, Regensburg. The partial specific volume at pH 7 was assumed to be 0.736 cm^3/g; for pH 2, it was corrected according to Durchschlag and Jaenicke (1982), using bovine serum albumin as a standard.

[^{35}S]Rubisco (~190 Ci·mol^{-1}) was synthesized and purified as previously described (Viitanen et al., 1992).

Rubisco activity was determined as previously described (Goloubinoff et al., 1989a). Further experimental details are given in the figure legends.

Results

Properties of Four Stable Conformational States

Native States, N_1 and N_2. The three-dimensional structure of Rubisco from *R. rubrum* has been determined to 1.7-Å

[2] In referring to specific amino acid residues, the single-letter code followed by the residue number is used. In the case of site-directed mutants, the substituted amino acid is given following the residue number. For example, the designation K168E refers to a mutation in which a glutamate has been substituted for the wild-type lysine at position 168.

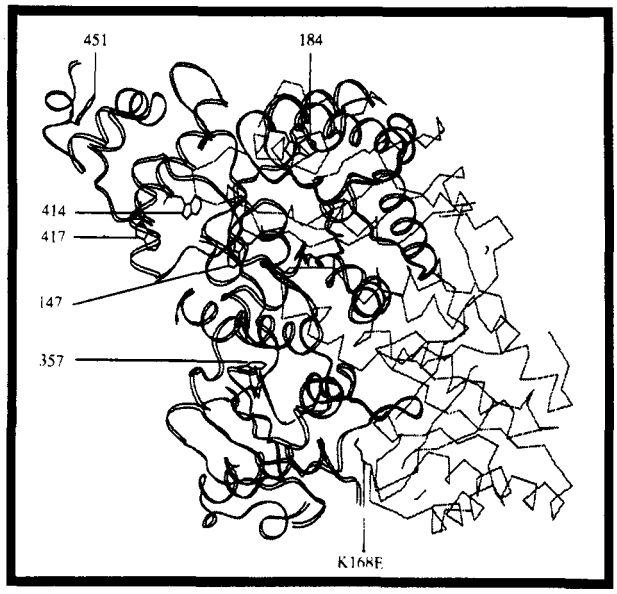


FIGURE 1: Native Rubisco dimer and the positions of the six tryptophan residues. Note that none of them is located at the interface between the subunits. Of the six residues, only W417 is in contact with more than one molecule of solvent (Schneider et al., 1990). Note also the position of K168 which forms a salt bridge (not shown) with E48 across the interface between the two subunits.

resolution (Schneider et al., 1990). Each identical subunit of the native dimer consists of three domains, the amino-terminal domain (residues 1–155), the $\alpha_8\beta_8$-barrel domain (residues 156–420), and a tail section (residues 421–466) of four α-helices (Figure 1). Genetic (Larimer et al., 1987) and structural analyses (Schneider et al., 1990) reveal that the dimer is the minimal catalytically active unit, a single active site containing amino acid residues from both subunits. The native dimer is stabilized by a number of polar interactions at the interface of the two subunits (Schneider et al., 1990). Among these, two salt bridges between the ϵ-amino group of K168 of one subunit and the carboxyl group of E48 of the other subunit are especially important. Neither K168 nor E48 is part of a secondary structural element. In the site-directed mutant K168E, these interactions are disrupted with the consequence that the mutant cannot form a homodimer (Mural et al., 1990). Instead the mutant forms a moderately stable, folded monomer, N_1, which is catalytically inactive. Importantly, Mural et al. (1990) were able to restore catalytic activity to this mutant by complementation in vivo. Since the complementing mutant was itself inactive, even as a homodimer, the appearance of catalytic activity can only have arisen by heterodimer formation. A single K168–E48 salt bridge is therefore sufficient to stabilize the subunit–subunit interaction. We have purified the mutant K168E to homogeneity (see Experimental Procedures) and have confirmed by in vitro complementation (see below) that it is monomeric.

Since the folded mutant monomer is capable of forming a heterodimer, when it is paired with a complementing monomer (see below), a comparison of its spectral properties with those of the folded wild-type dimer is valid. The far-UV CD spectra of the folded mutant monomer N_1 and the folded wild-type dimer N_2 are very similar (Figure 2C). Both spectra showed a broad region of equally negative ellipticity extending from about 208 nm to 224 nm and positive maxima at about 195 nm. Addition of 50 mM Na_2SO_4 did not significantly alter these spectra (data not shown). Using the method of Chen et al. (1972), we calculated the content of α-helix for both the mutant monomer and the wild-type dimer (Table I). These values, 45.6% and 42.6% for the N_1 and N_2 states, respectively,

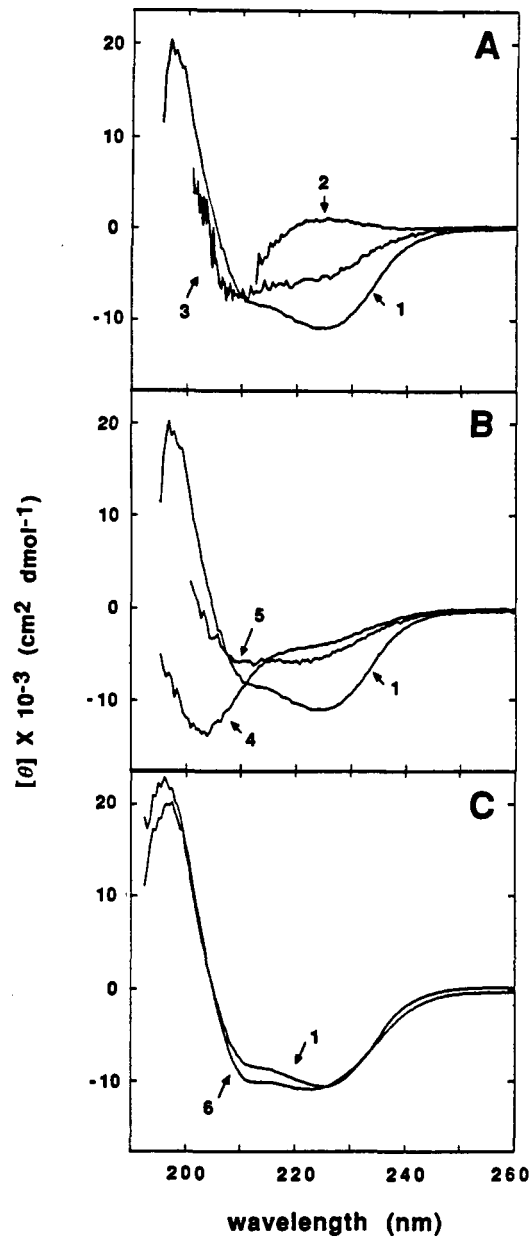


FIGURE 2: Circular dichroism spectra of Rubisco. (A) Curve 1, 5 μM native Rubisco-N_2 in 10 mM Tris-HCl, pH 7.6; curve 2, 5 μM unfolded Rubisco-UG_1 in 6 M guanidine hydrochloride and 10 mM Tris-HCl, pH 7.6; curve 3, partly folded Rubisco-I was prepared by diluting 250 μL of 8.4 mM Rubisco-UG_1 into 25 mL of 10 mM Tris-HCl, pH 7.6 at 4 °C. This solution was used to wash the cooled CD cell until no change in θ at a wavelength of 222 nm was observed. The Rubisco protomer concentration was calculated to be 65 nM using the determined amino acid composition of the solution in the CD cell. (B) Curve 1, 5 μM Rubisco-N_2 in 10 mM KCl; curve 4, 5 μM acid-denatured Rubisco-UA_1 in 10 mM HCl (pH 2); curve 5, partly folded Rubisco-A_1 in 10 mM HCl and 50 mM Na_2SO_4, at a final Rubisco protomer concentration of 80 nM. (C) Curve 1, 5 μM Rubisco-N_2 in 10 mM Tris-HCl, pH 7.6; curve 6, 5.5 μM Rubisco-N_1 (folded mutant K168E) in 10 mM Tris-HCl, pH 7.6. Spectra were recorded in a 1-mm path-length cell (5 μM solutions) or in a 5-cm path-length cell (65 and 80 nM solutions). All measurements were carried out at 4 °C using cooling jacketed cells. Data were collected at 0.5-nm intervals with time-averaging (15 s) on a AVIV CD spectrometer (Model 62 DS).

were slightly in excess of the 36.9% α-helical content derived from the three-dimensional structure (Schneider et al., 1990). We conclude that the creation of secondary structure is completed with the formation of the folded monomer.

R. rubrum Rubisco contains six tryptophan residues at positions 147, 184, 357, 414, 417, and 451 (Nargang et al.,

Table I: Properties of the Different Conformational States of Rubisco

form	condition	α-helix content (%)[a]	TRP fluorescence λ_{max} (nm)	Trp fluorescence intensity[b]	ANS fluorescence at 480 nm	weight average, molecular mass (kDa)
stable states						
native Rubisco (Rubisco-N_2)	pH 7.6, I = 0.01	42.6	332	100	2	95.2 ± 3.5
folded Rubisco monomer (Rubisco-N_1)	pH 7.6, I = 0.01	45.6	340	58	0	
unfolded Rubisco (Rubisco-UG_1)	pH 7.6, 4 and 6 M Gdn·HCl	0	350	48		
unfolded Rubisco (Rubisco-UA_1)	pH 2, I = 0.01	9.1	350	51	92	48.5 ± 4.0
partly folded Rubisco (Rubisco-A_1)	pH 2, 50 mM Na_2SO_4	27.1	340	81	45	
unstable state						
partly folded Rubisco (Rubisco-I_1)	pH 7.6, I = 0.01	29.7	340	78	9	

[a] The α-helical content was calculated from $f_H = -([\theta]_{222} + 2340)/30300$ (Chen et al., 1972). [b] Expressed as a percentage relative to the fluorescence intensity of Rubisco-N_2.

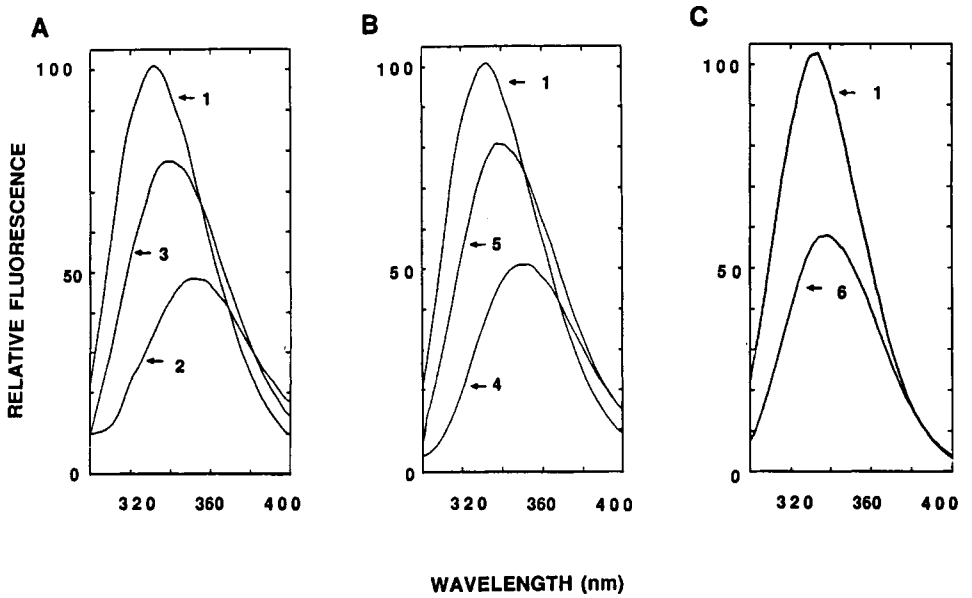


FIGURE 3: Tryptophan fluorescence emission spectra of Rubisco. (A) Curve 1, 75 nM native Rubisco-N_2 in 10 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, and 0.01% Tween-20; curve 2, 75 nM unfolded Rubisco-UG_1 in 6 M guanidine hydrochloride, 10 mM Tris-HCl, pH 7.6, 2 mM dithiothreitol, and 0.01% Tween-20; curve 3, partly folded Rubisco-I_1 prepared by diluting an aliquot of 8.4 μM Rubisco-UG_1 into 10 mM Tris-HCl, 2 mM dithiothreitol, and 0.01% Tween-20 at 4 °C to give a final concentration of 75 nM. The fluorescence emission spectrum of Rubisco-I_1 was recorded within 1 min of dilution. (B) Curve 1, 75 nM native Rubisco-N_2 in 10 mM KCl, 2 mM dithiothreitol, and 0.01% Tween-20; curve 4, 75 nM acid-denatured Rubisco-UA_1 in 10 mM HCl (pH 2), 2 mM dithiothreitol, and 0.01% Tween-20; curve 5, 75 nM partly folded Rubisco-A_1 in 10 mM HCl, 50 mM Na_2SO_4, 2 mM dithiothreitol, and 0.01% Tween-20. (C) Curve 1, 5.1 μM native Rubisco-N_2 in 10 mM Tris-HCl, pH 7.6; curve 6, 5.1 μM folded mutant monomer (Rubisco-N_1) in 10 mM Tris-HCl, pH 7.6. The tryptophan fluorescence emission was measured with excitation at 290 nm on a Perkin-Elmer LS-5B luminescence spectrometer. The temperature was controlled to 4 °C. The slit width for both excitation and emission was 5 nm, and the scan speed was 60 nm/min with a response time of 4.2 s. Similar results were obtained when 0.01% Tween-20 was omitted and the cuvette was rinsed with the protein solution prior to recording the spectrum.

1984). Their positions within the molecule are shown in Figure 1. These tryptophan residues are largely buried within the structure of the native dimer, only one (W417) of the six residues having contact with more than two molecules of water (Schneider et al., 1990). Significantly, none of the six tryptophan residues is located at the interface between the subunits (Figure 1). The fluorescence spectrum of the native wild-type dimer excited at 290 nm had an emission maximum at 332 nm (Figure 3C). This is significantly blue-shifted from the emission maximum of free tryptophan in aqueous solution (λ_{max} = 350 nm). This is consistent with the crystallographic data (Schneider et al., 1990) which show that the tryptophan residues in the native protein experience a hydrophobic environment.

As shown in Figure 3C, the tryptophan fluorescence spectrum of the mutant monomer, K168E, differed from that of the native, wild-type dimer. The emission maximum (λ_{max} = 340 nm) was red-shifted relative to the dimer, and the intensity was reduced (Table I). Since none of the six tryptophan residues are located at the subunit interface, the actual process of dimerization would not be expected to shield any of the tryptophan residues from the solvent. Nevertheless, the fluorescence spectrum of the folded mutant monomer suggests that the tryptophan residues are in a less hydrophobic and more mobile environment than they ultimately experience upon dimerization. This can be explained if we assume that the tight packing of the secondary structural elements and of the domains, typical of the native dimer, is more relaxed in the folded monomer.

The probe ANS fluoresces weakly in aqueous buffer solutions but becomes highly fluorescent when bound to the hydrophobic regions of proteins (Weber & Laurence, 1954; Stryer, 1965). However, little or no fluorescence change occurred upon adding the native dimer (Figure 4) or the native monomer (data not shown), indicating the absence of exposed hydrophobic regions. The failure of the native monomer to enhance the fluorescence of ANS was unexpected since crystallographic studies (Schneider et al., 1990) have established that the interactions at the interface between the subunits are in part hydrophobic in nature. Together the results of these fluorescence studies indicate that the folded monomer, when free in solution, adopts a different conformation from

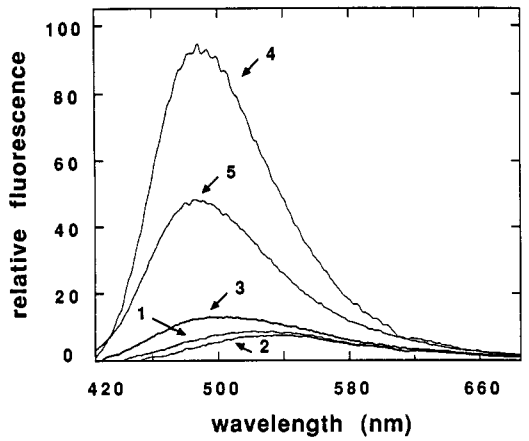


FIGURE 4: Fluorescence emission spectra of ANS in the presence of Rubisco. A solution of 82 μM 1-anilinonaphthalene-8-sulfonic acid (ANS, from Sigma) was incubated for 5 min at 4 °C in the presence of 83 nM Rubisco in the indicated conformational states. Curve 1, native Rubisco-N_2 in 10 mM Tris-HCl, pH 7.6; curve 2, ANS alone in 10 mM Tris-HCl, pH 7.6; curve 3, partly folded Rubisco-I_1 which had been prepared as described in the legend of Figure 2; curve 4, acid-denatured Rubisco-UA_1 in 10 mM HCl (pH 2); curve 5, partly folded Rubisco-A_1 in 10 mM HCl and 50 mM Na_2SO_4. The excitation wavelength was 400 nm and the temperature 4 °C. All other conditions were as described in the legend of Figure 3.

that observed in the native dimer. Similar conclusions have already been reached by Weber (1987) and Erijman and Weber (1991) following high-pressure dissociation studies of a number of oligomeric enzymes. These include the β_2 dimer of tryptophan synthase and enolase which, like Rubisco, possess a domain comprising an eight-membered $\alpha\beta$ barrel.

Rubisco-N_2 migrated as a single symmetrical band in the analytical ultracentrifuge with an $s_{20,w}$ value of 5.43 ± 0.14 S. The corresponding weight average molecular mass was 95.2 ± 3.5 kDa (Table I). This is a little less than the 101 kDa computed from the amino acid sequence (Nargang et al., 1984) and presumably reflects the fact that the native dimer has an ellipsoid shape with dimensions 45 Å × 70 Å × 105 Å (Branden et al., 1986).

Acid-Denatured States, UA_1 and A_1. The acid-denatured states of a number of proteins have recently been investigated (Arakawa et al., 1987; Kuwajima, 1989; Goto & Fink, 1989, 1990; Ikeguchi & Sugai, 1989; Goto et al., 1990a,b). Two acid-denatured states have been recognized, the UA state and the A state (Goto et al., 1990a). The UA state, typically observed at about pH 2 under conditions of low ionic strength, is extensively unfolded to conformations resembling those observed in chaotropes such as 6 M Gdn-HCl. Increasing the ionic strength at pH 2 causes the UA state to fold to a conformation, the A state, with properties akin to the so-called molten globule conformation observed for other proteins. Typically, A states are more compact and have a greater content of secondary structure than other unfolded states. The tryptophan residues characteristically experience a more hydrophobic environment in the A state than in other unfolded states. Finally, the A states of β-lactamase and apomyoglobin (Goto & Fink, 1990; Goto et al., 1990a,b) enhance the fluorescence of extrinsic probes such as ANS much more than do either the native or the UA states, indicating the existence of exposed hydrophobic regions.

The previously reported far-UV CD spectrum of acid-denatured Rubisco was determined under conditions of high ionic strength (0.1 M glycine hydrochloride, pH 3.0) (Goloubinoff et al., 1989). It was suggested that this corresponded to the so-called molten-globule or A state. Following others (Arakawa et al., 1987; Kuwajima, 1989; Goto & Fink, 1989, 1990; Ikeguchi & Sugai, 1989; Goto et al., 1990a,b), we have investigated the influence of ionic strength on the spectral properties of acid-denatured Rubisco. Both the far-UV CD spectrum (Figure 2B) and the tryptophan fluorescence spectrum (Figure 3B) of Rubisco at low ionic strength (10 mM HCl) were indicative of a substantially unfolded state. Thus, the far-UV CD spectrum of Rubisco, denatured in 10 mM HCl (Figure 2B), showed a broad band of negative ellipticity at 200–203 nm, typical of unfolded protein. However, compared with the spectrum of the Gdn·HCl-unfolded state, there was still sufficient negative ellipticity at 223 nm in the spectrum of the UA_1 state of Rubisco to suggest the presence of some residual structure (Table I). A similar observation has been made with the UA_1 states of apomyoglobin and ferricytochrome *c* (Goto et al., 1990a). The tryptophan fluorescence of Rubisco in 10 mM HCl (Figure 3B) was almost indistinguishable from that in 6 M Gdn·HCl and from that of free tryptophan, indicating an unfolded state. With emission maxima at 350 nm, these spectra were significantly red-shifted and quenched compared with the native state (Table I).

Upon increasing the ionic strength (with the addition of 50 mM Na_2SO_4), spectral changes consistent with a transition to an A_1 state were recorded (Figure 2B,B). The far-UV CD spectrum assumed a more nativelike form (Figure 2B), which is consistent with the formation of the A_1 state. The strongly negative band at 200–203 nm was replaced by a positive signal at these wavelengths. The ellipticity at 208 nm for the A_1 state at pH 2 was the same as that for the N_1 and N_2 states at pH 7.6. However, the α-helical content of the A_1 state (27.1%) was less than that of the native state (42.6%) (Table I). Addition of 50 mM Na_2SO_4 generated the A_1-state emission spectrum shown in Figure 3B. The fluorescence emission maximum of the A_1 state was the same as that of the folded mutant monomer, K168E, in the N_1 state. However, the fluorescence intensity is noticeably greater in the A_1 state (Figure 3 and Table I).

Figure 4 shows the fluorescence spectra of ANS in the presence of the UA_1 and A_1 states of Rubisco. Unlike the acid-denatured states of other proteins [e.g., β-lactamase and apomyoglobin (Goto et al., 1990a; Goto & Fink, 1989)], ANS fluorescence was more strongly enhanced by Rubisco in the UA_1 state than by Rubisco in the A_1 state (Figure 4). In both states, the emission maximum was similarly blue-shifted to 480 nm, indicating that the ANS was sequestered in a hydrophobic environment.

At pH 2.0 and low ionic strength, Rubisco-UA_1 sedimented as a monomer with a weight average molecular mass of 48.5 kDa (Table I). The sedimentation coefficient, $s_{20,w}$, was 1.60 ± 0.05 S. Since a globular protein of this size would be expected to have a sedimentation coefficient of between 3 and 4 S, the unusually low value of 1.6 S points to a strongly anisotropic shape for the UA_1 state. Attempts to determine the sedimentation behavior of the A_1 state were hampered by its propensity to aggregate (see below) at the concentrations necessary for its observation in the centrifuge.

Properties of the I_1 State, a Folding Intermediate

Previous work on the spontaneous folding of Rubisco has shown that the yield of reconstituted, active enzyme is highly dependent upon the concentration of protein and on the temperature (Goloubinoff et al., 1989a; Viitanen et al., 1990). In the absence of chaperonins, the unfolded protein only refolded to the native state under a restricted set of conditions (temperatures below about 10 °C and low protein concentrations). Beyond these conditions, irreversible aggregation occurred. Before investigating the spectral properties of the folding in-

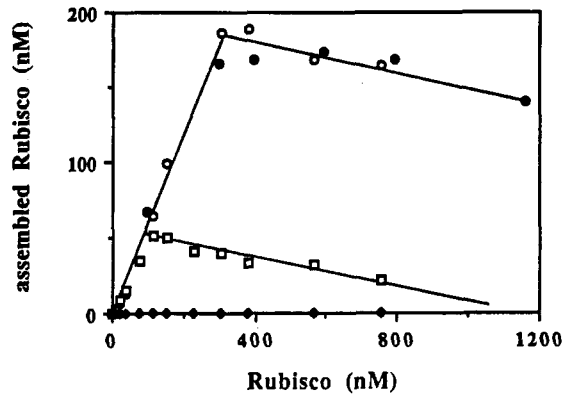


FIGURE 5: Stability of the I_1 and A_1 states of Rubisco as a function of concentration. The stability of Rubisco-I_1 as a function of concentration and temperature was determined as follows: Ten microliters of an appropriate concentration of Rubisco-UG_1 was diluted into 400 μL of a solution at the indicated temperature containing 50 mM Tris-HCl, 8.3 mM $MgCl_2$, 12 mM KCl, 7 μM bovine serum albumin, and 2 mM dithiothreitol, so as to generate the concentrations of Rubisco indicated on the horizontal axis. The solutions were incubated for 21 h at 4 (○), 15 (□), and 25 °C (♦) to allow spontaneous reconstitution of the enzyme. The reaction mixtures were brought to 25 °C, and an aliquot was analyzed for Rubisco activity. An aliquot of Rubisco-N_2 in 0.1 M Tris-HCl, pH 7.6, was diluted to a final concentration of 232 nM in the buffer described above and incubated for 21 h at 4 °C. The activity of this native control was used to compute the concentration of reconstituted enzyme (vertical axis). The stability of Rubisco-A_1 as a function of concentration was determined as follows: Four microliters of an appropriate concentration of Rubisco-UA_1 was diluted into 400 μL of 10 mM HCl containing 50 mM Na_2SO_4 at 4 °C so as to generate the concentrations of Rubisco indicated (●) on the horizontal axis, and the solutions were incubated for an additional 30 min at 4 °C. Next the samples were neutralized by dilution into a solution at 4 °C containing 50 mM Tris-HCl, pH 7.9, 8.3 mM $MgCl_2$, 12 mM KCl, 7 μM bovine serum albumin, and 2 mM dithiothreitol, so as to give a final concentration of 100 nM or less. (This procedure effectively converted any nonaggregated molecules in the A_1 state to the I_1 state, which, as shown with the protocol described above, reverted to the native state with high yield, provided the concentration was less than approximately 160 nM. This condition was met upon neutralizing the samples.) The samples were thereafter incubated at 4 °C for 23 h to permit spontaneous reversion to the native state. Next the samples were warmed to 25 °C, and an aliquot was analyzed for Rubisco activity as before.

termediate(s) or I state(s) of Rubisco, we sought to define the maximum concentration of Rubisco that was able to revert to the native state without sustaining losses due to aggregation. This information enabled us to maximize the spectral signals due to the I_1 state without having to be concerned about the presence of aggregates. Since the half-time for spontaneous refolding is about 5 h at 4 °C (Figure 6), it became possible to examine the properties of the I_1 state before significant reversion to the native states had occurred.

In the experiment shown in Figure 5, increasing quantities of unfolded Rubisco in 6 M Gdn·HCl were diluted (1:67) into solutions at 4, 15, and 25 °C and permitted to refold at those temperatures for 24 h. The samples were then assayed for catalytic activity at 25 °C. At 4 and at 15 °C, the absolute yield of refolded Rubisco was at first a linear function of the quantity of added unfolded protein (as one would expect if, instead, native protein had been added), but only up to a certain point, beyond which no further increases in activity were detected. On the contrary, beyond this point, a slight reduction in the absolute yield was evident at both temperatures. The relative yield of native enzyme in the linear phase corresponded to about 60–70% recovery. The missing 30% cannot be attributed to aggregation since the relative yield in the linear phase was independent of concentration and temperature.[3] Up to a well-defined concentration of the I_1 state, which we refer to as the critical aggregation concentration (CAC), the I_1 state remained monomeric (see below) and committed to folding to the native state. However, at concentrations of the I_1 state greater than the CAC, the yield of folded protein was diminished by an amount at least as large as the quantity in excess of the CAC (Figure 5). The Rubisco-I_1 in excess of the CAC was irreversibly lost as an aggregate, but that which remained was committed to folding to the native state as before. The CAC for the refolding of Rubisco was temperature-dependent. At 4 °C, it corresponded to about 160 nM refolded enzyme and at 15 °C to about 50 nM refolded protein while at 25 °C it was too low to be measured.

The propensity for the A states of α-lactalbumin (Kuwajima, 1989) and of β-lactamase (Goto & Fink, 1989) to undergo aggregation has been previously reported. As noted above, our attempts to determine the sedimentation behavior of Rubisco-A_1 were thwarted by its aggregation at the concentrations required for the analysis. To ensure that the spectral properties of Rubisco-A_1 were not complicated by aggregation, we systematically explored its propensity to aggregate as a function of concentration in a manner similar to that described for Rubisco-I_1 above. Varying concentrations of Rubisco were incubated for 30 min at pH 2 with 50 mM Na_2SO_4 at 4 °C. The samples were then neutralized and diluted to concentrations less than 100 nM, and the incubation was continued for a further 23 h at 4 °C. Molecules of Rubisco-A_1 which survived the acid bath without undergoing irreversible aggregation were thereby converted to Rubisco-I_1 and hence to the native state. It is apparent from Figure 5 that the propensity of Rubisco-A_1 to aggregate at pH 2.0 was similar to the propensity of Rubisco-I_1 to aggregate at neutral pH.

To explore the competition between aggregation and refolding to the native state, we exploited the ability of cpn60 to arrest the folding reaction. In the experiment shown in Figure 6, unfolded Rubisco was diluted into a solution at 4 °C to give a final concentration of 229 nM promoter, a value that was above the CAC. The protein was then permitted to partition to aggregates or to spontaneously refold to the native state. At the times indicated, excess cpn60 was added to arrest the folding reaction. After 25 h, the samples were brought to 25 °C, supplemented with either cpn10 alone or cpn10 and MgATP, incubated for a further 90 min, and assayed for Rubisco activity. What was being measured by such a protocol? The activity in the samples which ultimately received only cpn10 reflected the quantity of refolded enzyme existing at the time of the cpn60 quench. The activity in the samples which ultimately received both cpn10 and MgATP reflected the sum of the quantity of refolded enzyme existing at the time of the cpn60 quench *plus* the quantity of folding intermediate trapped by cpn60 at the time of the quench and subsequently converted to active enzyme upon incubation with cpn10 and MgATP. The difference between these two measurements therefore reflected the quantity of folding intermediate existing at the time of the quench. At time zero, i.e., immediately after dilution, there existed no folded Rubisco. On the other hand, excess cpn60 trapped a species, corresponding to about 210 nM, which when supplemented with cpn10 and MgATP reverted to the native state. (This is indicated by an arrow in Figure 6.) At concentrations above the CAC, this cpn60-

[3] Experiments with [^{35}S]Rubisco-I_1 indicate that some of this loss can be attributed to interactions with the surfaces of the containment vessels. Alternatively, intramolecular misfolding events which are irreversible on the time scale in question may contribute to the shortfall.

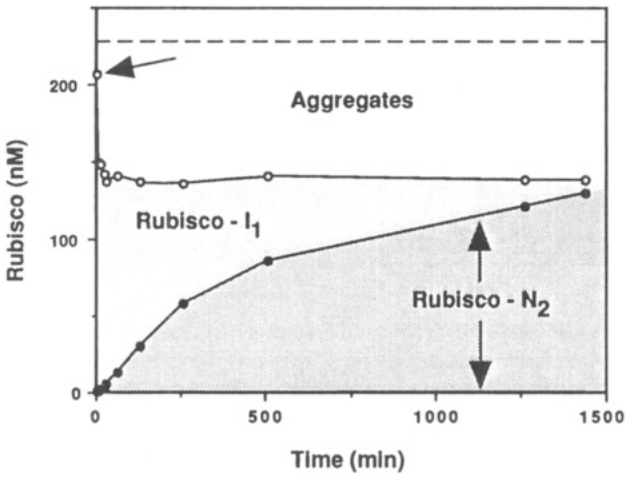


FIGURE 6: Rubisco-I_1 is substrate for the chaperonin-dependent reconstitution reaction. The experiment was performed at 4 °C as follows: For each time point, 6 μL of Rubisco-UG_1 was diluted into 400 μL of as solution containing 50 mM Tris-HCl, pH 7.6, 8.3 mM $MgCl_2$, 12 mM KCl, 7 μM bovine serum albumin, and 2 mM dithiothreitol to give a final Rubisco protomer concentration of 229 nM. At appropriate times, the reconstitution reaction was terminated by adding cpn60 to a final protomer concentration of 3.2 μM, after which the reaction mixture was further incubated at 4 °C. After 24 h, the solutions were brought to 25 °C. Two aliquots (112 μL each) of each reaction mixture were supplemented with cpn10 to give a final protomer concentration of 8.3 μM. To one series of aliquots was added MgATP to a final concentration of 3.3 mM. The reaction mixtures were incubated at 25 °C for 90 min and subsequently analyzed for Rubisco activity. The amount of refolded Rubisco is shown for reactions with (○) and without (●) ATP. The concentration of refolded Rubisco was determined by reference to the activity of native Rubisco of known concentration. The dashed line represents the concentration of unfolded Rubisco initially diluted into the folding reaction mixture. The arrow at time zero indicates the quantity of refolded Rubisco achieved when 3.2 μM cpn60 (protomer) was already present in the solution into which the unfolded Rubisco was diluted.

trappable species rapidly declined in concentration until the CAC was reached. In the experiment shown in Figure 6, the CAC was reached within 5 min, indicating that the commitment to aggregation is rapid. The species which remained was fully committed to refolding to the native state. Alternatively, it could be trapped by cpn60 and subsequently converted to the native state by the addition of cpn10 and MgATP. This species became resistant to interaction with cpn60 with kinetics indistinguishable from those associated with the appearance of the native state.

The stability of the I_1 state at concentrations below the CAC and the sluggishness with which it reverted to the native state at 4 °C (Figure 6) afforded us a chance to characterize some of its spectral properties, unencumbered by the complications arising due to aggregation.

Spectral Properties of the I_1 State. Solutions of the I_1 state of Rubisco were generated by diluting unfolded Rubisco into buffer solutions at 4 °C, so as to establish concentrations less than the CAC. These were analyzed within 30 min, before significant reversion to the native state had occurred. The tryptophan fluorescence spectrum of the I_1 state at pH 7.6 was very similar to both that of the A_1 state at pH 2.0 (Figure 3A) and that of the folded, mutant monomer N_1 (Figure 3C). The emission maxima of these three states were the same, and the degrees of quenching, relative to the native dimer, were similar. This does not mean that each of the six tryptophan residues is experiencing the same microenvironment in each of the three states, since the spectra are the sum of the contributions of each of the six residues which may be different. Nevertheless, the spectrum of the I_1 state was established within a matter of seconds, and only changed very slowly (i.e., on a time scale of hours) as the native dimer was formed (data not shown). This indicates that the formation of the I_1 state was very rapid, relative to the rate at which the native state is formed.

The far-UV CD spectrum of the I_1 state (Figure 2A) is very nearly the same as that of the A_1 state (Figure 2B), indicating a similar degree of secondary structure formation. These secondary structural elements may or not be identical in the two states. Judged from the ellipticity at 222 nm (Table I), the α-helix content of the I_1 state was about 27%, or about two-thirds that of the native state. Regardless of the identity of the α-helices present in the I_1 state, the transition to the folded N_1 state must be accompanied by the formation of some additional elements of secondary structure.

The ANS fluorescence spectrum in the presence of the I_1 state (Figure 4) is qualitatively similar to the spectra observed in the presence of both acid-denatured states (Figure 4) in that the emission maximum is blue-shifted to about 470 nm. Accordingly, the transition to the folded N_1 state must also be accompanied by the burial of the hydrophobic regions exposed in the I_1 state.

It was not possible to determine the oligomeric state of Rubisco-I_1 by analytical ultracentrifugation, since the minimal concentration necessary for such an analysis was well in excess of the CAC. We therefore turned to in vitro complementation analysis to address this question.

In Vitro Complementation Studies. Chaperonins certainly facilitate the folding of monomeric proteins, such as rhodanese (Mendoza et al., 1991) and dihydrofolate reductase (Viitanen et al., 1991). To determine whether the species of Rubisco recognized by cpn60 was monomeric or dimeric, we exploited the folded monomer K168E and the knowledge that it could be complemented in vivo with the appropriate subunit (Mural et al., 1990). Use was made of the site-directed mutant N111V as the complementing mutant. Since this mutant has the wild-type residues K168 and E48, it forms a stable homodimer. However, it cannot catalyze the fixation of CO_2 on account of the Asn to Val substitution at residue 111 within the active site (Figure 7). In theory then, if these two mutant, inactive monomers are permitted to combine so as to form a heterodimer, a single functional, wild-type active site should be created. When the native dimeric-complementing mutant N111V was mixed with the native monomeric mutant K168E, essentially no Rubisco activity resulted (Figure 8). This indicates that the native homodimer N111V did not undergo appreciable subunit exchange with K168E, since this would have led to the formation of an active heterodimer. The dimeric N111V was next unfolded in 10 mM HCl and used to prepare a binary complex with $[cpn60]_{14}$. This was then mixed with a solution containing the already folded monomer K168E plus cpn10 and MgATP to complete the reconstitution of N111V. Catalytically active heterodimers were formed. The relative yield of active heterodimers was about 70% of the maximum possible when the ratio of folded mutant (K168E) monomer to complementing refolded mutant (N111V) was 12:1 (Figure 8). The relative yield of active heterodimer decreased as the ratio of [K168E] to [N111V] was reduced, presumably due to the formation of the more stable, but inactive N111V homodimer. From these results, it may be concluded that in order to form an active heterodimer, the complementing homodimer must first be dissociated into its subunits, for example, by acid denaturation. It therefore follows that the UA_1 state, the I_1 state, and the product released by the chaperonin are monomeric.

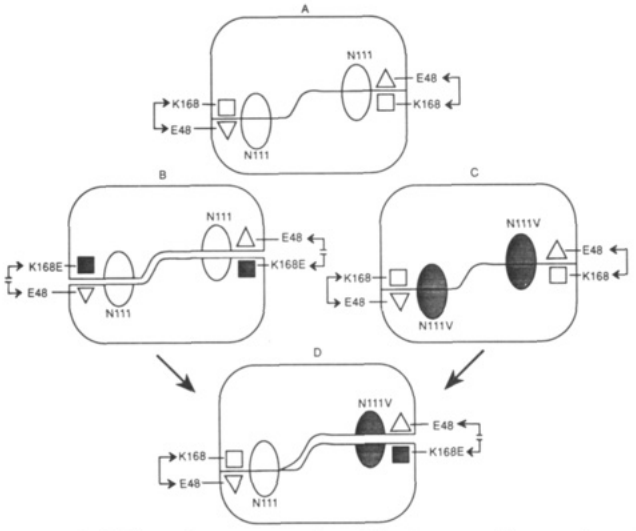


FIGURE 7: Schematic representation of in vitro complementation of the monomeric mutant K168E with a subunit derived from the dimeric mutant N111V. The species A represents the catalytically active wild-type homodimer $(E48/N111/K168)_2$ with two E48–K168 salt bridges at the interface between the subunits. The species B represents the catalytically inactive mutant monomer E48/N111/K168E. This is unable to form a stable homodimer since it lacks the interfacial salt bridges. The species C represents the catalytically inactive mutant homodimer $(E48/N111V/K168)_2$. Although it possesses both interfacial salt bridges, it is inactive on account of the active-site mutation N111V. The species D represents an active heterodimer (E48/N111V/K168)(E48/N111/K168E) composed of a subunit from each mutant. It possesses one interfacial salt bridge and one functional active site.

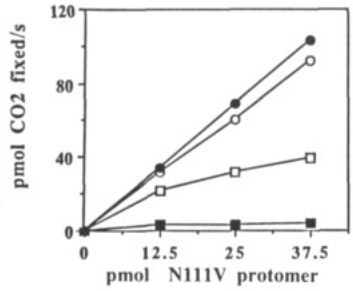


FIGURE 8: In vitro complementation of the inactive folded mutant monomer K168E by a subunit derived from the inactive mutant dimer N111V. The complementing mutant N111V was either denatured in 10 mM HCl or left in its native state in 10 mM KCl. The indicated quantities of acid-denatured N111V (open squares) or of native N111V (closed squares) were added to 230 μL of an ice-cold solution of 114 mM Tris-HCl, pH 7.9, 11.4 mM KCl, 11.4 mM magnesium acetate, 22.7 mM dithiothreitol, 3.61 μM cpn10 (protomer), and 1.54 μM cpn60 (protomer). Controls containing the same quantities of acid-denatured wild type (open circles) or native wild type (closed circles) were similarly prepared. The samples were next brought to 25 °C and supplemented with 150 pmol of the folded mutant monomer K168E. Reconstitution was initiated by the addition of 0.5 μmol of ATP. After 40 min, Rubisco activity was determined as previously described (Goloubinoff et al., 1989). No $^{14}CO_2$ fixation was observed in controls containing either native N111V or native K168E (data not shown).

Susceptibility to Proteolysis. A characteristic feature of the nonnative states of many proteins is their enhanced sensitivity to proteolytic digestion. Thus, even when bound to cpn60, the nonnative states of mouse dihydrofolate reductase (Viitanen et al., 1991; Martin et al., 1991) and of rhodanese (Martin et al., 1991) are more readily digested than their native states. As shown in Figure 9, this enhanced sensitivity extends to the form of Rubisco bound to cpn60. Since the I_1 state of Rubisco, free in solution, is similarly sensitive to proteolysis as the cpn60-bound material, sensitivity to proteolysis is not conferred by binding to cpn60 but is rather a property of the nonnative state.

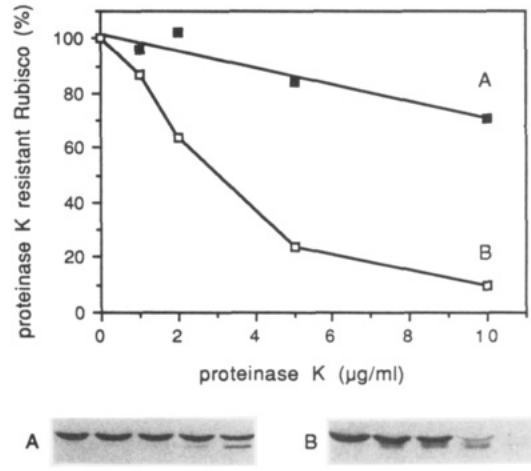
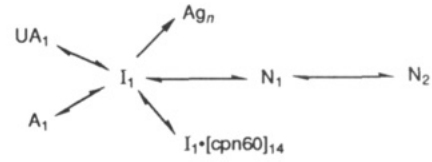


FIGURE 9: Protease sensitivity of native Rubisco-N_2 or Rubisco-I_1 in the presence of cpn60. (A) ^{35}S-Rubisco-N_2 ($\sim$190 Ci mol^{-1}) was rapidly diluted 52-fold to a final concentration of 380 nM protomers in 0.1 M Tris-HCl, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.89 μM $[cpn60]_{14}$, and the mixture was incubated at 23 °C for 10 min. (B) ^{35}S-Rubisco-N_2 was acid-denatured in 10 mM HCl (pH 2) for 1 h at 23 °C, then rapidly diluted 8-fold to a final concentration of 850 nM protomer in 0.1 M Tris-HCl, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1.7 μM $[cpn60]_{14}$, and incubated at 23 °C for 10 min. Both samples were then chilled on ice and treated with the indicated quantities of proteinase K for 10 min. Proteolysis was terminated with 10 mM phenylmethanesulfonyl fluoride, and the samples were analyzed by SDS–PAGE/fluorography using 12.5% gels. Radioactive bands corresponding to intact Rubisco have been quantitated in the upper part of the figure while the original fluorographs are shown below.

Scheme II



Discussion

Analyses of the pathway of folding of Rubisco revealed four stable conformational states: two acid-denatured states (UA_1 and A_1) and two folded states, the folded monomer N_1 and the native, biologically active dimer N_2. Spectral and kinetic analyses establish the existence of a fifth conformational state, I_1, which is unstable, however. Under narrowly defined conditions (i.e., at permissive temperatures and at concentrations below the critical aggregation concentration), the I_1 state converts spontaneously but slowly to the native states, N_1 and N_2 (Viitanen et al., 1990). At restrictive temperatures, the I_1 state is especially prone to irreversible aggregation. These states are related to one another in the manner shown in Scheme II.

A common feature of several proteins is the existence of two distinct acid-denatured states, UA and A (Goto & Fink, 1989, 1990; Goto et al., 1990a,b). The UA state is largely unfolded and is favored by conditions of low ionic strength, whereas the A state, favored by high ionic strength, contains significant secondary structure. Spectroscopic analyses by 2D NMR of the A states of α-lactalbumin (Baum et al., 1989) and of cytochrome *c* (Jeng et al., 1990) have revealed the presence of nativelike elements of helical structure. The A state is believed to be structurally similar to the so-called molten globule conformation posited to be formed as an intermediate

during the folding of many proteins (Ptitsyn, 1987; Kuwajima, 1989). The limited spectral analyses of the acid-denatured states of Rubisco point to the existence of these two states. Of particular interest is the similarity between the far-UV CD and tryptophan fluorescence spectra of the A_1 and the I_1 states of Rubisco, the former a stable species observable at pH 2 and at high ionic strength and the latter formed transiently as a folding intermediate at neutral pH. The two states also show the same propensity to aggregate. These properties suggest that the A_1 and I_1 states may share a common conformation. However, although the α-helical content of both states is similar and intermediate between the completely unfolded and native states, this does not necessarily mean that the same α-helices are present in both A_1 and I_1 states. On the other hand, it is not unreasonable to speculate, given the existence of nativelike α-helices in the A states of α-lactalbumin and cytochrome c, that this may be so. Regardless of the identity of the α-helices, both the A_1 and I_1 states lack the ordered teriary structure associated with the native dimer.

Another common property of both the I_1 and A_1 states of Rubisco is their propensity to undergo aggregation above a defined concentration which we refer to as the CAC. Aggregation is believed to be the result of illegitimate hydrophobic interactions between surfaces transiently exposed during the folding process (Mitraki & King, 1989). The transition from a monomeric state I_1 or A_1, committed to folding, to an irreversibly aggregated state Ag_n (Scheme II) occurs over a very limited range of concentration. The sharpness of this transition reminds one of micellation, which is also thought to involve hydrophobic interactions. These hydrophobic interactions, and hence aggregation, can be reduced by lowering the folding temperature.

Recently, in a study of the competition between refolding and aggregation of folding intermediates of lysozyme, Goldberg et al. (1991) demonstrated that the rate of formation of the species of lysozyme which undergo aggregation is rapid (i.e., aggregation is complete within 1 min). However, the folding intermediates become resistant to aggregation much more slowly, essentially as they are converted to the native state. The behavior of the I_1 state of Rubisco is similar in many respects. Thus, when introduced at concentrations greater than the CAC, the I_1 state rapidly aggregates in the absence of cpn60 until the CAC is reached (Figure 6). In the presence of cpn60, however, the I_1 state can be substantially trapped in the form of a binary complex (arrowed data point at time zero in Figure 6). The folding intermediates which remain at or below the CAC nevertheless continue to be capable of interacting with cpn60. The capacity to interact with cpn60 is lost with kinetics indistinguishable from those associated with the appearance of the native states.

All of the unfolded (UA_1) and partly folded (A_1 and I_1) states bind ANS more strongly than either of the native states N_1 and N_2, pointing to the presence of exposed hydrophobic surfaces. The illegitimate interaction of these hydrophobic surfaces probably accounts for the propensity of the I_1 state to aggregate. Aggregation is prevented by the presence of a molar excess of $[cpn60]_{14}$ with the formation of a stable binary complex. However, the formation of the binary complex also halts the transition to the native states. The simplest interpretation of these results is that $[cpn60]_{14}$ interacts directly with the I_1 state. However, the conformational state of I_1 free in solution may not be the same as that bound to cpn60. Martin et al. (1991) have concluded that the species of dihydrofolate reductase and rhodanese bound to cpn60 lacks organized tertiary structure. They refer to these species as "molten globules", although no evidence was presented that these bound species possessed any secondary structure, perhaps the most important characteristic of molten globules. In contrast, after a kinetic analysis of the interaction of the folding intermediates of lactate dehydrogenase with cpn60, Badcoe et al. (1991) specifically concluded that cpn60 did not interact with the molten globule state. The species of lactate dehydrogenase defined as the molten globule state [U_3 of Badcoe et al. (1991)] was the second folding intermediate in a kinetically defined folding pathway ($U_1 \rightarrow U_2 \rightarrow U_3 \rightarrow$ native dimer), involving two unimolecular steps and a final bimolecular step to give the native state. In some respects, however, the U_3 state of lactate dehydrogenase resembles the N_1 state of Rubisco. Both are distinguished from their respective native states by differences in the tryptophan fluorescence spectra, both are monomeric, and neither appears able to interact with cpn60 (data for Rubisco-N_1 not shown).

The folding pathway of dimeric Rubisco also resembles that of dimeric aspartate aminotransferase (Herold & Kirschner, 1990). Two monomeric folding intermediates of aspartate aminotransferase, designated M* and M, were described along the pathway $U \rightarrow M^* \rightarrow M \rightarrow$ native dimer. The species M* and M appear to be equivalent to the I_1 and N_1 states of Rubisco, respectively. Herold and Kirschner (1990) refer to the M* species of aspartate aminotransferase as a molten globule state. However, it is not known if this molten globule state of aspartate aminotransferase can interact with cpn60.

In an attempt to define the structural element recognized by cpn60, Landry and Gierasch (1991) have examined the interaction of a number of small peptides with cpn60, using 2D NMR. They have shown that a synthetic peptide, which in native rhodanese is α-helical, adopts an α-helical conformation while bound to cpn60. [Cpn60 is known to interact with nonnative state(s) of rhodanese (Mendoza et al., 1991).] In free solution, this helical species is poorly populated, suggesting that cpn60 stabilizes a minor species which already possesses secondary structure. If this result can be obtained with a wider variety of peptides with the propensity to form α-helices, it would strengthen the notion that cpn60 interacts with folding intermediates rather than with completely unfolded states.

These last results also point to a limitation in the present analyses, namely, the likely existence of equilibria between all of the species along the folding pathway. On the basis of some recent rapid CD studies (Kuwajima et al., 1987, 1991), unfolded states are quite likely to be in rapid equilibrium with the collapsed or molten globule state. Thus, our conclusion that $[cpn60]_{14}$ interacts directly with the I_1 state of Rubisco needs to be accompanied with the caveat that poorly populated conformational states in equilibrium with the I_1 state may also interact with cpn60. It is further known that conformers of the native states of some proteins exist [e.g., dihydrofolate reductase (Viitanen et al., 1991) and pre-β-lactamase (Laminet et al., 1990)] which can be recognized by cpn60. Incubation of the native states of these proteins with a slight molar excess of $[cpn60]_{14}$ results in the progressive loss of catalytic activity which may, however, be recovered by the addition of MgATP. In effect, cpn60 acts as a trap for the nonnative states of these proteins which exist in equilibrium with the native states on a time scale which is experimentally accessible. Clearly, the I_1 state of Rubisco must exist in some equilibrium with the N_1 state. However, our attempts to demonstrate a stable complex by mixing cpn60 with the folded mutant monomer have so far failed (data not shown). It may be that the rate of the N_1 to I_1 transition is so slow and the equilibrium so

disfavorable that the I_1 state of Rubisco remains experimentally inaccessible from the N_1 state. Further analyses using increased concentrations of chaotropes to destabilize the I_1 and N_1 states with a view to resolving some of the above uncertainties are currently in progress.

ACKNOWLEDGMENTS

We thank Dr. F. C. Hartman and Dr. F. W. Larimer of the Biology Division, Oak Ridge National Laboratory, for making available to us the plasmid encoding the monomeric mutant Rubisco K168E and our colleague Dr. Steven Gutteridge for the gift of the purified mutant N111V. Tom Webb purified the proteins used in this study, and Karen Bacot provided technical assistance.

REFERENCES

Arakawa, T., Hsu, Y.-R., & Yphantis, D. A. (1987) *Biochemistry 26*, 6545–6552.

Badcoe, I. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P., & Clarke, A. R. (1991) *Biochemistry 30*, 9195–9200.

Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) *Biochemistry 28*, 7–13.

Branden, C. I., Schneider, G., Lindqvist, Y., Andersson, I., Knight, S., & Lorimer, G. H. (1986) *Philos. Trans. R. Soc. London B 313*, 359–365.

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmidt, F. X., & Kiefhaber, T. (1991) *Biochemistry 30*, 1586–1591.

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

Chen, Y.-H., Yang, J. T., & Marinez, H. (1972) *Biochemistry 11*, 4120–4131.

Creighton, T. E. (1990) *Biochem. J. 270*, 1–16.

Durchschlag, H., & Jaenicke, R. (1982) *Biochem. Biophys. Res. Commun. 108*, 1074–1079.

Ellis, R. J., & van der Vies, S. M. (1991) *Annu. Rev. Biochem. 60*, 321–347.

Erijman, L., & Weber, G. (1991) *Biochemistry 30*, 1595–1599.

Gatenby, A. A., Castleton, J. A., & Saul, M. W. (1981) *Nature 291*, 117–121.

Goldberg, M. E., Rudolph, R., & Jaenicke, R. (1991) *Biochemistry 30*, 2791–2797.

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

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

Goto, Y., & Fink, A. L. (1989) *Biochemistry 28*, 945–952.

Goto, Y., & Fink, A. L. (1990) *J. Mol. Biol. 214*, 803–805.

Goto, Y., Calciano, L. J., & Fink, A. L. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577.

Goto, Y., Takahashi, N., & Fink, A. L. (1990b) *Biochemistry 29*, 3480–3488.

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., Georgopoulos, C., Hendrix, R. W., & Ellis, R. J. (1988) *Nature 333*, 330–334.

Hendrix, R. W. (1979) *J. Mol. Biol. 129*, 375–392.

Herold, M., & Kirschner, K. (1990) *Biochemistry 29*, 1907–1913.

Ikeguchi, M., & Sugai, S. (1989) *Int. J. Pept. Protein Res. 33*, 289–297.

Ito, K., Sato, T., & Yura, T. (1977) *Cell 11*, 551–559.

Jeng, M.-F., Englander, W., Elove, G. A., Wand, A. J., & Roder, H. (1990) *Biochemistry 29*, 10433–10437.

Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.

Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.

Kuwajima, K., Yamaya, H., Miwa, S., Sugai, S., & Nagamura, T. (1987) *FEBS Lett. 221*, 115–118.

Kuwajima, K., Garvey, E. P., Finn, B. E., Matthews, C. R., & Sugai, S. (1991) *Biochemistry 30*, 7693–7703.

Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C., & Pluckthun, A. (1990) *EMBO J. 9*, 2315–2319.

Landry, S. J., & Gierasch, L. M. (1991) *Biochemistry 30*, 7359–7362.

Larimer, F. W., Lee, E. H., Mural, R. J., Soper, T. S., & Hartman, F. C. (1987) *J. Biol. Chem. 262*, 15327–15329.

Lorimer, G. H., & Hartman, F. C. (1988) *J. Biol. Chem. 263*, 6468–6471.

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., & Hartl, F.-U. (1991) *Nature 352*, 36–42.

Mendoza, J. A., Rogers, E., Lorimer, G. H., & Horowitz, P. M. (1991) *J. Biol. Chem. 266*, 13044–13049.

Mitraki, A., & King, J. (1989) *Bio Technology 7*, 690–697.

Mural, R. J., Soper, T. S., Larimer, F. W., & Hartman, F. C. (1990) *J. Biol. Chem. 265*, 6501–6505.

Nargang, F., McIntosh, L., & Somerville, C. R. (1984) *Mol. Gen. Genet. 193*, 220–224.

Niyogi, S. K., Foote, R. S., Mural, R. J., Larimer, F. W., Mitra, S., Soper, T. S., Machanoff, R., & Hartman, F. C. (1986) *J. Biol. Chem. 261*, 10087–10092.

Olsson, B., & Olsson, I. (1986) in *Electrophoresis '86* (Dunn, M. J., Ed.) pp 399–401, VCH Verlag, Weinheim, Germany.

Pierce, J., & Gutteridge, S. (1986) *Appl. Environ. Microbiol. 49*, 1094–1100.

Pierce, J., & Reddy, G. S. (1986) *Arch. Biochem. Biophys. 245*, 483–493.

Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273–293.

Schloss, J. V., Phares, E. F., Long, M. V., Norton, I. L., Stringer, C. D., & Hartman, F. C. (1982) *Methods Enzymol. 90*, 522–528.

Schneider, G., Lindqvist, Y., & Lundqvist, T. (1990) *J. Mol. Biol. 211*, 989–1008.

Somerville, C. R., & Somerville, S. (1984) *Mol. Gen. Genet. 193*, 214–219.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.

Taguchi, H., Konishi, J., Ishi, N., & Yoshida, M. (1991) *J. Biol. Chem. 266*, 22411–22418.

Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., & Lorimer, G. H. (1990) *Biochemistry 29*, 5665–5671.

Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H., & Gatenby, A. A. (1991) *Biochemistry 30*, 9716–9723.

Viitanen, P. V., Gatenby, A. A., & Lorimer, G. H. (1992) *Protein Sci.* (in press).

Weber, G. (1987) in *High Pressure Chemistry and Biochemistry* (van Eldick, R., & Jonas, J., Eds.) pp 401–420, D. Reidel Publishing Co., Dordrecht, The Netherlands.

Weber, G., & Lawrence, D. J. R. (1954) *Biochem. J. 56*, xxxi.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–310.