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The Dimerization of Folded Monomers of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase*[S]

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Shen Luo, Zheng-Yu Wang, Masayuki Kobayashi, and Tsunenori Nozawa‡

From the Department of Biomolecular Engineering, Faculty of Engineering, Center for Interdisciplinary Research, Tohoku University, Sendai 980-8579, Japan

Spontaneous refolding and reconstitution processes of dimeric ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Rhodospirillum rubrum* have been investigated using size-exclusion high performance liquid chromatography (HPLC), spectroscopic, and activity measurements. When the unfolded Rubisco in guanidine hydrochloride is diluted at 4 °C, a folding intermediate (Rubisco-I) is rapidly formed, which remains in an unstable monomeric state and gradually develops into folded monomer (Rubisco-M) at 4 °C but undergoes irreversible aggregation at 25 °C. Refolding of Rubisco-I to Rubisco-M is a very slow process, taking about 20 h for 70% conversion at 4 °C. Rubisco-M is stable at 4 °C and is capable of forming an active dimer spontaneously when incubated at a temperature higher than 10 °C. The dynamic dimerization process has been measured in a temperature range of 4–35 °C by HPLC, and the results demonstrate that the dimerization is strongly facilitated by the temperature. It is found that dithiothreitol is essential for the spontaneous reconstitution of Rubisco.

Ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco)[1] catalyzes the carboxylation of ribulose 1,5-bisphosphate, giving rise to two molecules of 3-phosphoglycerate. This is the major entry point for CO_2 into the biosphere. The enzyme also catalyzes the oxygenation of the same substrate, giving rise to a molecule of 3-phosphoglycerate and a molecule of phosphoglycolate (1). From a structural viewpoint Rubisco may be isolated in either of two forms, I and II. In higher plants, algae, and most other photosynthetic organisms, the enzyme is a complex molecule consisting of eight large (L, $M_r \sim 55{,}000$) and eight small (S, $M_r \sim 14{,}000$) subunits, forming an L_8S_8 (form I) complex. In the photosynthetic bacterium *Rhodospirillum rubrum*, on the other hand, the enzyme is a dimer of large subunits, L_2 (form II), each of relative molecular weight 55,000 (2).

The assembly of Rubisco is of special biological interest for two main reasons: first, the enzyme represents a model system in the study of chloroplast biogenesis, because its subunits are synthesized in different cellular compartments. Second, it has not been possible to reconstitute any higher plant Rubisco from fully dissociated subunits (3, 4). Studies of *in vitro* assembly have primarily focused on the simple dimeric enzyme from *R. rubrum*. It has been shown that accessory proteins or "molecular chaperones" (5) are required to assist the assembly of unfolded monomers or nascent polypeptide chains along a pathway that favors the formation of the correctly folded and properly arranged active dimer (6, 7). It is well established that when dimeric Rubisco from *R. rubrum* is synthesized in *Escherichia coli*, successful assembly requires fully functional chaperonin proteins: chaperonin 10 (cpn10) (GroES) and cpn60 (GroEL) (6). It has also been demonstrated that *in vitro* reconstitution of the catalytically functional dimeric Rubisco from an unfolded, biologically inert state depends on the presence of both chaperonins and MgATP (7, 8). The chaperonin-facilitated refolding of Rubisco can be dissected into two steps. In the first step, a non-native state of the protein, folding intermediate (Rubisco-I), is stabilized by forming a binary complex with cpn60. In the second step, cpn10 couples the K^+-dependent hydrolysis of ATP to release the folded monomers of the target protein from cpn60. In the absence of chaperonins, the unfolded Rubisco could also revert spontaneously to the native state but only under a restricted set of conditions: low temperatures and low protein concentrations. Beyond these conditions, irreversible aggregation occurred (8, 9).

Little is known about the dimerization process of the folded monomers, which is the last step in the reconstitution of native Rubisco. In most experiments the refolding process of unfolded protein to a folded monomer and the assembling process of the folded subunits to a multisubunit complex can not be distinguished, and it is difficult to produce an initial state where the folded monomers remain stable. In this study, we demonstrate that the spontaneous reconstitution to a fully active Rubisco can occur with a clearly defined two-stage process from the completely unfolded state. The unfolded Rubisco monomers from *R. rubrum* first undergo slow refolding and remain in a stable monomeric state at 4 °C. Dimerization of the folded monomers is initiated by elevating temperature to above 10 °C. Rate of formation and final yield of the dimeric Rubisco is strongly dependent on the temperature in addition to the incubation time.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Assay—The purple nonsulfur bacterium *R. rubrum* was grown in a culture medium containing butyrate as a carbon source as described previously (10, 11). Rubisco from *R. rubrum* was prepared according to a reported procedure (11) with some modifications. Extraction and purification of the enzyme were conducted using a buffer (pH 8.0, 4 °C) containing 20 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 50 mM $NaHCO_3$ (TEMMB buffer).

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‡ To whom correspondence should be addressed: Tel.: 81-22-217-7277; Fax: 81-22-217-7279; E-mail: nozawa@biophys.che.tohoku.ac.jp.

[1] The abbreviations used are: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); HPLC, high performance liquid chromatography; Gdn-HCl, guanidine hydrochloride; CD, circular dichroism; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; CAC, critical aggregation concentration.

The fraction precipitated in 30–60% saturated $(NH_4)_2SO_4$ was resuspended in TEMMB buffer and chromatographed by a DEAE-Sephacel column (2.5 × 25 cm, Amersham Pharmacia Biotech), followed by a gel-filtration column (Sephacryl S-200 HR, 2.5 × 100 cm, Amersham Pharmacia Biotech). The eluted fractions containing Rubisco were chromatographed again by a DEAE-Sephadex A-50 column (1 × 30 cm, Amersham Pharmacia Biotech) and finally by a size-exclusion HPLC column (TSKgel G3000SW, 2.15 × 30 cm, Tosoh). The enzyme was purified to homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified Rubisco was rapidly frozen in liquid nitrogen and stored at −85 °C until use. The concentration of Rubisco was determined by the bicinchoninic acid (BCA) method (12), giving an absorption coefficient of $\epsilon_{280\,nm} = 7.20 \times 10^4\ M^{-1}\ cm^{-1}$ for Rubisco protomer. The molecular weight of Rubisco protomer was taken as 55,000 from light scattering measurement (2). Ribulose 1,5-bisphosphate carboxylase activity and the CO_2/O_2 specificity of Rubisco were determined by ^{31}P NMR method as described in the previous papers (13, 14). Ribulose 1,5-bisphosphate was purchased from Sigma Chemical Co.

Unfolding and Reconstitution of Rubisco—The starting material for spontaneous reconstitution experiments was prepared by denaturing native Rubisco in guanidine hydrochloride (Gdn-HCl) solution. A small aliquot of Rubisco was dissolved in 6 M Gdn-HCl solution of 0.1 M Tris-HCl, pH 7.5, 4 mM EDTA, and 0.1 M dithiothreitol (DTT) to a protomer concentration of 13.9 μM. The unfolding was continued for at least 30 min at 25 °C. Refolding was achieved by diluting 30 μl of the unfolded Rubisco into 1.47 ml of the standard refolding buffer containing 0.1 M Tris-HCl, pH 7.5, 4 mM EDTA, 5 mM DTT, and 10 mM $MgCl_2$ at 4 °C. To determine the degree of refolding in a particular experiment, an aliquot of native Rubisco was treated by the same procedure, except that Gdn-HCl was omitted. Dimerization was initiated by elevating the temperature of the refolded samples in an incubator for a period of time without changing the solution composition. Further experimental details are given in the figure legends.

Size-exclusion HPLC Analysis—All HPLC measurements were performed in a 4 °C chamber. Buffers used for elution were degassed and filtered through a Millipore 0.22-μm filter. The samples were centrifuged at 17,500 × *g* for 5 min at a desired temperature prior to injection to remove any possible precipitate. Typically, 100 μl of Rubisco sample in the native, refolded, or reconstituted state was loaded on a size-exclusion HPLC column (TSKgel $G3000SW_{XL}$, 0.78 × 30 cm, Tosoh) equilibrated and eluted with 50 mM MOPS buffer (pH 7.0) containing 0.2 M Na_2SO_4. The flow rate was 0.5 ml/min, and elution of the protein was monitored by measuring absorbance at 280 nm using an ultraviolet detector (UV-8020, Tosoh). The standard molecular weight marker (Calibration Proteins II for chromatography) was purchased from Roche Molecular Biochemicals Biochemica (Germany).

Spectroscopic Measurements—Far-UV circular dichroism (CD) spectra were measured in a wavelength range of 215 and 260 nm with a Jasco J-720W spectropolarimeter at 25 °C. Data were collected at 1.0-nm intervals with accumulation of 15 times. Autofluorescence spectra were recorded with an RF-5300PC spectrofluorophotometer (Shimadzu) using a 1- × 1-cm quartz cuvette at 25 °C. The excitation wavelength was set at 290 nm. Fluorescent probe 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt (bis-ANS) was purchased from Sigma Chemical Co. The fluorescence experiments using bis-ANS were carried out under conditions similar to those for autofluorescence except that the excitation wavelength was set at 390 nm.

RESULTS

Fig. 1 shows the HPLC elution profiles of native and dissociated Rubisco using a size-exclusion column. The elution buffer contained 0.2 M Na_2SO_4, because the silica gel-based column is a "nonideal size-exclusion" column and could not be calibrated in a low ionic strength buffer due to nonspecific ionic interactions of charged groups of the gel matrix with the protein (15, 16). Native Rubisco, *N*, was eluted at 17 min as a single peak. The elution time corresponded to an apparent molecular weight of 116,000 as calculated from the calibration (Fig. 1, *inset*), confirming a dimeric form of the enzyme. The dissociated Rubisco monomer (after refolding for 60 min at 4 °C), *M*, was eluted at 18.2 min, corresponding to a monomeric form with a M_r of 58,000. Because the time required for refolding and dimerization is much longer (~8 h for 35% activity recovery) than the time used for the HPLC analysis, the whole

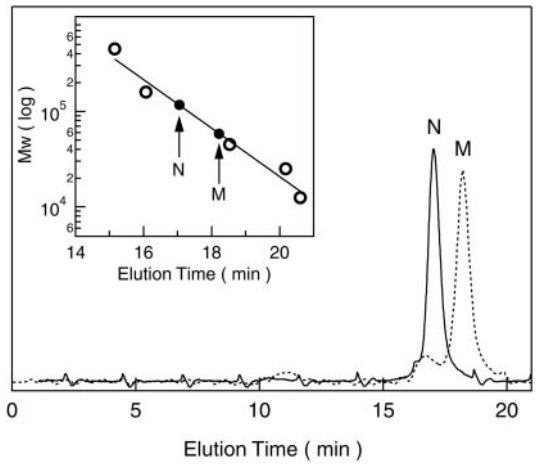

FIG. 1. **Determination of molecular weight of Rubisco in different states by size-exclusion HPLC at 4 °C.** Rubisco was passed through a $TSKgel\ G3000SW_{XL}$ HPLC column (0.78 × 30 cm) at 4 °C as described under "Experimental Procedures". Elution of protein was monitored by measuring the absorbance at 280 nm. The elution profiles of Rubisco are denoted as *N* (native) and *M* (dissociated monomer state), and the M state of Rubisco was prepared by diluting unfolded Rubisco into a standard refolding buffer at 4 °C for 60 min. The experimental conditions for unfolding and reconstitution are described under "Experimental Procedures". *Inset*, molecular weight calibration curve. The column was calibrated with the following molecular weight standards (from *left* to *right*): ferritin (M_r = 450,000), aldolase (M_r = 158,000), albumin from hen egg (M_r = 45,000), chymotrypsinogen A (M_r = 25,000), and cytochrome *c* (M_r = 12,500). Elution peaks of native (*N*) and monomeric (*M*) Rubisco are marked by *arrows* in the curve.

reconstitution process can be monitored by the HPLC system.

Fig. 2*A* shows the time changes of HPLC elution profiles of the refolding Rubisco species at 4 °C after dilution of the guanidine-denatured protein. All the profiles exhibited similar feature with a major elution peak at 18.2 min, indicating that the Rubisco existed in a monomeric state at this temperature. There was a small peak with a slightly shorter elution time, which was thought to correspond to a dimeric form, but this peak did not develop over 24 h. Fig. 2*B* shows the effect of incubation time at 4 °C (first stage) on the dimer formation. The subsequent incubation at 25 °C (second stage) was conducted for 30 min for all samples. The dimeric peaks increased significantly, and correspondingly, the monomeric peaks decreased. Further extension of the second stage incubation time to 2 h at 25 °C resulted in nearly complete conversion to the dimer species (Fig. 2*C*). The remainder of the monomers that cannot be converted even for longer incubation time at 25 °C indicates the existence of a small amount of misfolded species. Activity recovery was found to have good correlation with the amount of dimers as calculated from the peak areas of the chromatograms (Fig. 2*C*). It should be mentioned that reducing agent DTT is essential for both refolding and reconstitution of the active Rubisco. All experiments of refolding and reconstitution were performed in the buffer containing 5 mM DTT.

Comparing the peak areas of dimer and monomer in Fig. 2, one may note that the peak areas were almost unchanged for the refolding species at 4 °C within 24 h (Fig. 2*A*), whereas the total peak areas changed remarkably upon the first stage refolding time at 4 °C (Fig. 2, *B* and *C*). About two-thirds of the total species were undetected for the samples with shorter refolding times (2–2.5 h) in the first stage at 4 °C before elevating the incubation temperature to 25 °C. Because no additional peaks were detected during 0–16 min in the elution profiles, the loss of species may be attributed to the formation of large aggregates, which were finally removed by the centrifugation before injecting into the HPLC column. With the increasing refolding time at 4 °C, the total peak area increased,

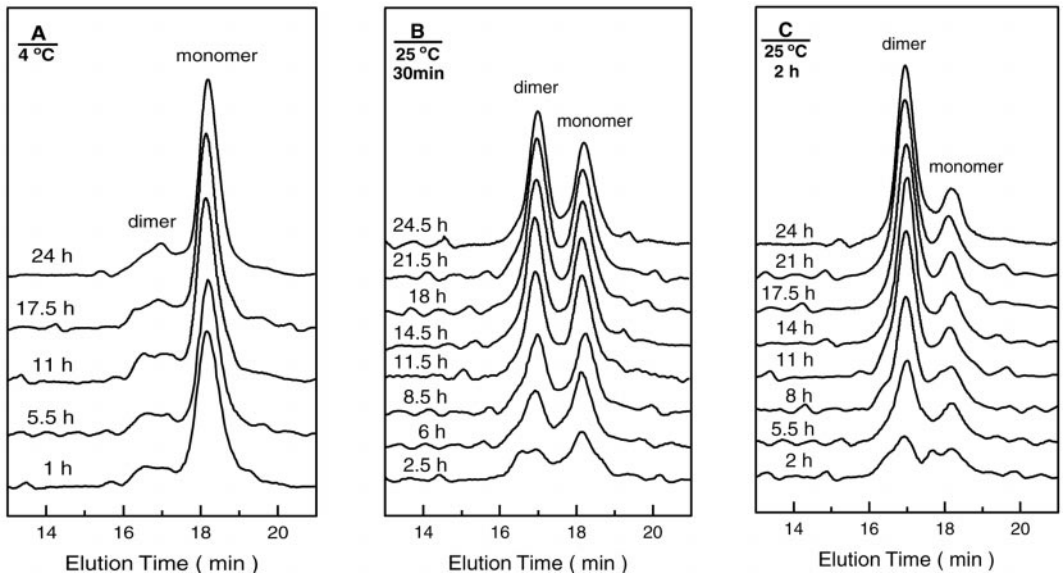

FIG. 2. **Analysis of refolding Rubisco species by size-exclusion HPLC during reconstitution.** Rubisco (13.9 μM protomer) was first unfolded by Gdn-HCl at 25 °C for 60 min, then diluted 50-fold into a standard refolding buffer as described under "Experimental Procedures." The refolding time in the first stage at 4 °C is indicated on each chromatogram. *A*, chromatograms of the samples with only first stage treatment for various incubating times at 4 °C. After centrifugation at 4 °C for 5 min, a 100-μl sample was injected into the HPLC column at 4 °C. *B*, chromatograms of the samples incubated for various times at 4 °C in the first stage and subsequently treated at 25 °C for 30 min in the second stage. The samples were centrifuged at 25 °C for 5 min, then a 100-μl solution was injected into the column at 4 °C. *C*, similar to the samples described in *B* except that the incubating time was 2 h in the second stage.

indicating that only the folded monomers formed in the first stage are capable of forming dimers subsequently at higher incubation temperatures. Therefore, there were at least three species in the refolding solution. At 4 °C, most species exist as monomers but in two states: one as the refolding intermediates (Rubisco-I) and the other as folded monomers (Rubisco-M). Both of the monomeric species gave essentially the same elution time in the chromatogram. In the early stage of refolding, most of the monomeric species can be considered as Rubisco-I, which is unstable at 4 °C and tends to form irreversible aggregates at 25 °C. With the increasing refolding time at 4 °C, Rubisco-I was slowly folded into a more stable Rubisco-M. Complete conversion from Rubisco-I to Rubisco-M took about 24 h under the experimental conditions of this study. Rubisco-M is stable at 4 °C and undergoes spontaneous dimerization by incubation at temperatures higher than 4 °C to form native dimer (Rubisco-N).

Fig. 3 shows the temperature dependence of second stage dimerization of the Rubisco-M formed at 4 °C for 24 h. Higher temperature led to higher formation rate and yield of the dimer species. The temperature effect was most significant between 15 and 30 °C, as more than 90% of reconstitutable Rubisco-M was converted into dimers within 30 min of incubation at 30 °C. These results clearly demonstrate that dimerization of the Rubisco is triggered by elevation of temperature and the rate of dimer formation and final yield are strongly dependent on the temperature. The reconstituted Rubisco gave the same CD and autofluorescence spectra, along with the CO_2/O_2 specificity factor as the native Rubisco (supplementary material), indicating that all structural and functional characteristics have been fully recovered.

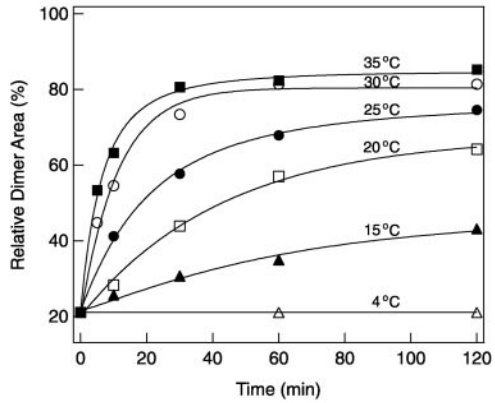

FIG. 3. **Temperature dependence of dimerization for the folded Rubisco monomers.** Rubisco was unfolded and refolded under the same conditions as described in the legend of Fig. 2. All the samples were first refolded at 4 °C for 24 h, and then followed by incubation at the temperatures indicated in the figure. Aliquots of the solution were centrifuged for 5 min at the same temperature and analyzed with HPLC at 4 °C. Percentages of the dimers formed were calculated as ratios of dimeric peak areas to the total peak areas (dimer + monomer) from the chromatograms.

DISCUSSION

HPLC technique has been extensively applied to study the dissociation and reconstitution processes of oligomeric proteins (15, 17–20). We demonstrated in this study that the spontaneous reconstitution of active Rubisco is a sequential process in which three distinct states can be observed using the size-exclusion HPLC. On the basis of our observations, the pathway of the reconstitution of dimeric Rubisco from a completely unfolded state (Rubisco-U) may be described as follows. First, the Gdn-HCl-denatured Rubisco is rapidly converted to an unstable intermediate state, Rubisco-I, by dilution. Second, refolding of the Rubisco-I to folded monomers, Rubisco-M, is a very slow process that takes about 24 h for completion at 4 °C. We could not directly observe the difference between Rubisco-I and Rubisco-M from the chromatogram, because both species eluted at the same retention time. However, we were able to indirectly evaluate the proportion of the two species from the subsequent dimerization process, because only Rubisco-M is capable of forming an active dimer when elevating the incubation temperature. The last step is the dimerization of Rubisco-M to form the native dimeric Rubisco (Rubisco-N). We further showed that the three states can be controlled by

changing either time or temperature of incubation. Proportion of Rubisco-M to Rubisco-I is determined by the incubation time at 4 °C, whereas the proportion of Rubisco-N to Rubisco-M is strongly dependent on the temperature in addition to the incubation time.

Several factors are found to affect the spontaneous refolding and reconstitution processes of Rubisco. Previous studies reported that a "critical aggregation concentration" (CAC) exists in the reactivation of Rubisco (9). Below the CAC, Rubisco-I can spontaneously develop into the native state (reactivation). However, at concentrations higher than the CAC, the Rubisco-I undergoes irreversible aggregation. The aggregation occurs rapidly (21) and proceeds until the concentration of Rubisco-I falls to the CAC (9). In this study, the CAC was determined to be about 0.9 μM (protomer concentration of Rubisco-U), and we also observed that the formation of aggregates was promoted by increasing the concentration of Rubisco-I above the CAC (data not shown).

Temperature is another important factor in the Rubisco refolding (8). Because the CAC decreases quickly as temperature increases (9), higher temperatures promote the irreversible aggregation of Rubisco-I. Aggregation is believed to result from irregular hydrophobic interaction between the Rubisco-I molecules on the surfaces exposed (22). We conducted a fluorescence experiment using the hydrophobic fluorophore bis-ANS (23–25) and observed that there was more than a 10-fold increase in the fluorescence intensity for the refolding species incubated for 1 h at 4 °C, as compared with the native Rubisco. This indicates the presence of a large region of exposed hydrophobic surface formed in the early stage of refolding. Further kinetic measurements on the binding of bis-ANS to protein revealed that refolding of Rubisco at 4 °C was a very slow process and the fluorescence decreased to a level near to that of native Rubisco when the Rubisco-M was further incubated at 25 °C (supplemental material).

It should be emphasized that DTT plays an essential role in whole processes from denaturing to reconstitution of the Rubisco. When Rubisco was unfolded in 6 M Gdn-HCl in the absence of DTT followed by refolding and reconstitution procedures, no spontaneous reactivation or very little recovery of the activity was detected. There are ten cysteine residues in *R. rubrum* Rubisco (26), and x-ray crystallographic studies have shown that the enzyme is devoid of disulfide bond (27). Therefore, the effect of DTT may be explained in terms of protection of formation of the disulfide bonds that lead to misfolding during unfolding and refolding processes. Reversible dimer-monomer dissociation of Rubisco from *R. rubrum* was investigated using hydrostatic pressure (28). Full reversibility after dissociation was observed to depend on the presence of small ligands, such as Mg^{2+} and $NaHCO_3$.

It has been well established that the reconstitution of active Rubisco from unfolded polypeptides is facilitated by the molecular chaperones, such as GroEL and GroES of *E. coli*, and MgATP (6–9). In previous studies, a site-directed mutant, K168E, which exists as a stable monomer, was often used to characterize the "native" monomeric state (9), corresponding to the Rubisco-M of this study, in connection with the role of chaperones in the interaction with folding intermediates. The Rubisco in this state was shown to have a similar secondary structure content but less ordered tertiary structure than that of the native dimer. We demonstrated in this study that the stable Rubisco-M species can be spontaneously formed at 4 °C without involvement of chaperones and be identified by HPLC analysis in its intact state.

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