Dithiothreitol decreases the thermal stability and unfolding cooperativity of ribulose-1, 5-bisphosphate carboxylase/oxygenase*

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Abstract Plant rubisco consists of eight large subunits (55 kD) encoded by chloroplast gene and eight small subunits (15 kD) encoded by nuclear gene. There are abundant cysteine residues that do not form disulfide bonds in native rubisco. Differential scanning calorimetry has been used to study some plant rubisco and suggested an irreversible two-state denaturation due to the high cooperativity in subunits. By comparing the data from circular dichroism, fluorescence, differential scanning calorimetry, SDS electrophoresis, and activity assays in the absence or presence of DTT, we suggest that the formation of disulfide bonds in subunits during the early thermal unfolding may increase the thermal stability and the thermal unfolding cooperativity of rubisco.

Keywords: DTT, rubisco, thermal unfolding, cooperativity, disulfide bond.

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco, EC 4.1.1.39) is a key enzyme in photosynthesis[1]. It has been extensively and intensively investigated on catalysis mechanism, activity regulation, crystal structure, genetic manipulation, and unfolding and assembly[2~5]. Differential scanning calorimetry (DSC) has been used to study some plant rubisco and suggested an irreversible two-state denaturation due to the high cooperativity within rubisco molecules[6~9]. Also the spectroscopy study on spinach rubisco demonstrated that the tertiary and/or quaternary structural change occurs before the DSC phase transition during thermal unfolding[10]. In general, hydrophobic interaction, hydrogen bond, and liganded interface are main contributions to the cooperativity of protein folding/unfolding[11]. Rubisco contains abundant aromatic residues (Spinach rubisco has 21 Phe, 19 Tyr and 8 Trp in its large subunit as well as 6 Phe, 10 Tyr and 4 Trp in its small subunit) that can provide more hydrophobic interfaces and hydrogen bonds than other residues. This may be one of the reasons that rubisco cooperatively unfolds during thermal denaturation. Interestingly, there are also abundant cysteine residues (Spinach rubisco has 9 Cys in its large subunit and 4 Cys in its small subunit) that do not form disulfide bonds in native rubisco. A question is whether the formation of disulfide bonds can contribute to the cooperativity during the thermal unfolding of rubisco. The free sulfhydryl of dithiothreitol (DTT) can prevent other sulfhydryl from oxidation and so DTT is usually used as a protective agent for cysteine in protein. If rubisco is heated in the presence of DTT, then the probability of the formation of disulfide bonds between cysteines will be significantly reduced and the thermal unfolding may be different from that in the absence of DTT. In this paper, circular dichroism (CD), fluoresence, differential scanning calorimetry (DSC), SDS electrophoresis, and activity assays were performed on the thermal unfolding of spinach rubisco in the absence or presence of DTT.

1 Materials and methods

1.1 Materials and rubisco purification

DTT, SDS, Hepes, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma. Sephadex G200, G25, and DEAE-Sephadex A25 were purchased from Pharmacia. All other chemicals were local products of analytical grade. Rubisco was purified from fresh spinach as reported method[12] and stored in $(NH_4)_2SO_4$ and N_2 saturated buffer (5 mmol/L DTT, pH7.6) at 4 ℃. Before assay, some stored rubisco was put through a Sephadex G25 column equilibrated with 25 mmol/L Hepes-KOH

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buffer (pH 8.3) to remove $(NH_4)_2SO_4$ and DTT. The concentration of rubisco was calculated from the absorbance at 280 nm with $A^{1\%} = 16.4$ cm·L/mol[13].

1.2 CD measurements

CD measurements were performed by a Jasco-715 spectropolarimeter. The temperature was controlled with a circulating water bath of which the thermocouple was close to the sample cell. The CD value at 222 nm was monitored to indicate the conformational change of rubisco when the sample was heated (1 ℃/min) to and incubated at a desired temperature. If the CD value at 222 nm kept constant, far UV wavelength scan was done. In all CD experiments, the concentration of rubisco in 25 mmol/L Hepes-KOH buffer (pH 8.3) was 0.2 mg/mL, and the lightlength of the sample cell was 0.1 cm.

1.3 Fluorescence measurements

Intrinsic emission spectra excited at 295 nm (only exciting Trp) for spinach rubisco (0.04 mg/mL) were recorded by a Hitachi F-850 spectrofluorometer in the absence or presence of 1 mmol/L DTT. The temperature was controlled as the CD measurements. Rubisco in 25 mmol/L Hepes-KOH buffer (pH 8.3) was heated from 25 ℃ to a desired temperature at 1 ℃/min, and then incubated at this temperature. When the fluorescence intensity at 330 nm was constant at the desired temperature, which indicated the equilibrium of the conformational change of rubisco, the emission wavelength scan was done.

1.4 DSC measurements

Differential scanning calorimetry measurements for spinach rubisco in the absence or presence of 3 mmol/L DTT were conducted with a Sateram Micro DSC III at a heating rate of 0.5 ℃/min. The concentration of rubisco was 8 mg/mL and the matrix was 25 mmol/L Hepes-KOH buffer (pH 8.3). The temperature range of the thermogram was from 20 to 90 ℃.

1.5 Activity assays

Rubisco, in native or heat-treated in the absence or presence of DTT (1.5 mmol/L), was incubated with activating mixture at 25 ℃ for 30 min. The activating mixture was 25 mmol/L Hepes-KOH buffer (pH 8.3) containing $NaHCO_3$ and $MgCl_2$. The final concentrations of rubisco, $NaHCO_3$, and $MgCl_2$ were 1 mg/mL, 20 mmol/L, and 10 mmol/L, respectively. Reaction mixture was 25 mmol/L Hepes-KOH buffer (pH 8.3) containing about 1 mmol/L RuBP, 10 mmol/L $MgCl_2$, 20 mmol/L $NaHCO_3$, and 10 mmol/L carbonic anhydrase. After 0.1 mL activated rubisco solution was added into 0.9 mL reaction mixture in the sample cell, the absorbance at 280 nm was immediately monitored by the Hitachi U-3010 spectrophotometer[14]. The reaction temperature was held at 25 ℃ controlled by a circulating water bath.

1.6 Electrophoresis experiments

Spinach rubisco (0.1 mg/mL) in the absence or presence of 1.5 mmol/L DTT was heated and then SDS was added into the sample to reach the concentration of 1%. The procedure for heating rubisco was the same as in the fluorescence measurement, and the fluorescence intensity at 330 nm was also monitored to indicate whether the conformational change reached the equilibrium. SDS polyacrylamide gel electrophoresis was carried out according to the standard protocol. The concentrations for the accumulating and separating gels were 3% and 12% respectively.

2 Results

2.1 CD spectra

The far UV spectra that reflected the secondary structure of spinach rubisco did not change from 25 to 45 ℃ but changed increasingly from 45 to 60 ℃, abruptly from 60 to 65 ℃ until complete unfolding (Fig. 1(a)). The change of CD value at 222 nm can mainly reflect the change of the á-helix scale of a protein and so indicates the trend of the change of a protein secondary structure. The temperature scan at 222 nm for spinach rubisco revealed the same changing tendency as the far UV CD spectra (Fig. 1(b)).

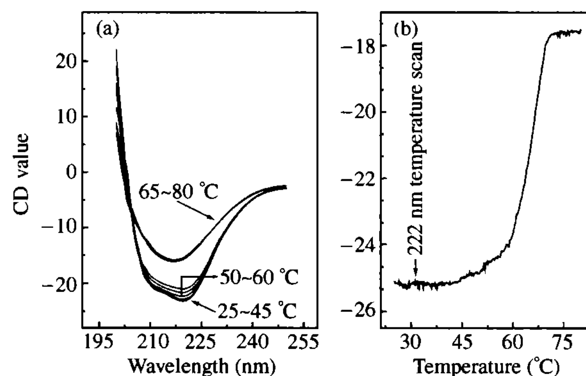

Fig. 1. Far UV CD spectra at different temperatures from 25 to 80 ℃ with a step of 5 ℃ (a) and temperature scan at 222 nm (b).

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2.2 Fluorescence

Tryptophan is useful to sensitively indicating the conformational change of a protein. The emission spectra excited at 295 nm (only exciting Trp) were recorded at different desired temperatures in the absence (Fig.2(a)) or presence (Fig.2(b)) of 1 mmol/L DTT. As well known, temperature is an extinction factor for fluorescence, so the spectral shift instead of the change of fluorescence intensity was analyzed by a sensitive method reported[15]. Since the spectral shift causes the fluorescence intensity to change oppositely most in two steep regions, particularly at 320 nm and 365 nm, the fraction of I_{320}/I_{365} can be sensitively indicative of the spectral shift. In the absence of DTT, the value of I_{320}/I_{365} decreased with temperature slowly going up from 25 to 45 ℃ with an almost constant region (35 ~ 45 ℃), rapidly from 45 to 60 ℃, and abruptly from 60 to 65 ℃ (Fig. 2(c), 1). However, in the presence of 1 mmol/L DTT, the value of I_{320}/I_{365} decreases with temperature much faster from 25 to 60 ℃, and especially, the constant region disappeared (Fig. 2(c), 2).

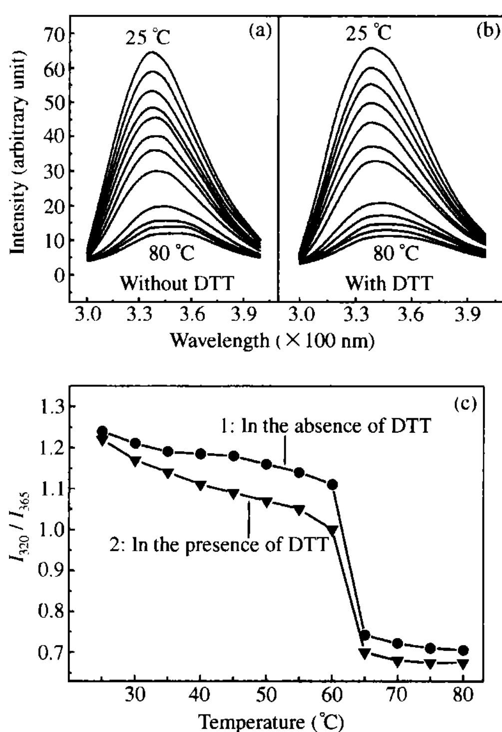

Fig. 2. Fluorescence spectra excited at 295 nm at different temperatures from 25 to 80 ℃ with a step of 5 ℃. (a) In the absence of DTT; (b) in the presence of 1 mmol/L DTT; (c) spectral shifts by plotting I_{320}/I_{365} against temperature.

2.3 Differential scanning calorimetry (DSC)

In the absence of DTT, the thermogram of DSC for spinach rubisco showed one approximatively symmetrical endotherm peak (Fig. 3, 1). This is almost the same as previously reported[6~9]. On the contrary, DSC gave a different thermogram for spinach rubisco in the presence of 3 mmol/L DTT (Fig. 3, 2). Although the main endotherm peak was primarily unchanged, a slow liquidation process occurred from 45 to 60 ℃.

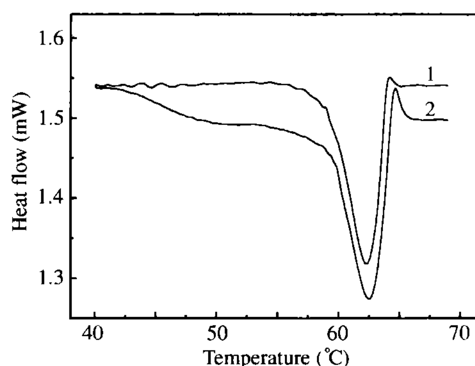

Fig. 3. DSC thermograms with a heating rate of 0.5 ℃/min. (1) In the absence of DTT; (2) in the presence of 3 mmol/L DTT.

2.4 Activity assay

Rubisco activity can be expressed as the rate of RuBP consumption. RuBP has an absorption peak at 280 nm with an extinction factor of about 50 cm/mol, and this property can be employed to determine rubisco activity by continuously measuring the absorbance decrease of the reaction system at 280 nm[14]. Here the activity is directly expressed as the change rate of the absorbance in the first three minutes. If the activity of control (not heat-treated) is defined as 1, then the restored activity for heated rubisco is a fraction of control. The plots of the fractions against temperature show that the activity loss rate of the heated rubisco in the presence of DTT was much higher than that in the absence of DTT when the temperature exceeded 45 ℃ (Fig. 4).

2.5 SDS electrophoresis

The results of SDS polyacrylamide gel electrophoresis for the heat-treated rubisco in the absence or presence of DTT are shown in Fig. 5. When the samples did not contain DTT (Fig. 5(a)), the bands for the small subunits of spinach rubisco were clear and uniform from 35 to 70 ℃. This demonstrated

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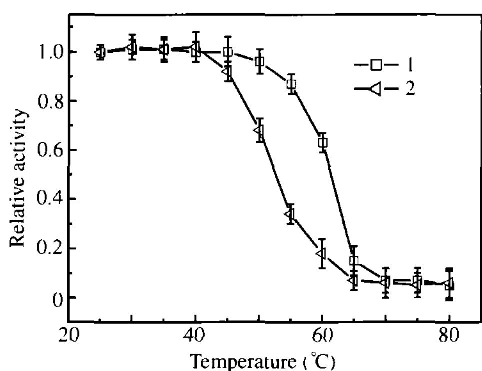

Fig. 4. Restoration of rubisco heat-treated in the absence (1) or presence (2) of 1.5 mmol/L DTT. All assays were repeated three times.

that the small subunits was not involved in forming disulfide bonds and were easily dissociated into monomers by SDS. But the bands for the large subunits gradually got faint from 45 ℃ and vanished at 70 ℃. In addition, the bands that should be the polymers of large subunits got gradually thick from 45 to 70 ℃ at the interface between the accumulating and separating gels. When the samples contained 1.5 mmol/L DTT (Fig. 5(b)), the bands for the large and small subunits were clear and uniform from 25 to 70 ℃, and there were not any bands at the interface between the accumulating and separating gels.

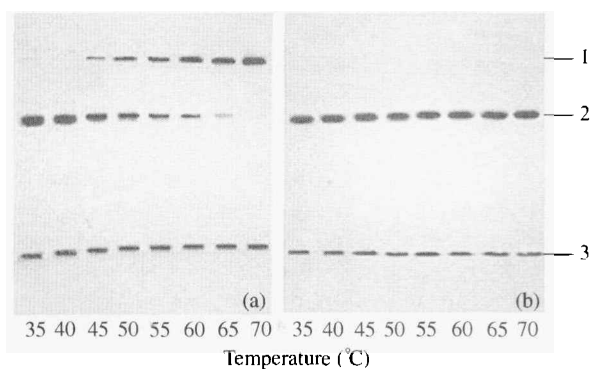

Fig. 5. SDS-PAEG (12%) for rubisco (0.1 mg/mL). (a) Heating SDS; (b) DTT heating SDS. 1, the interface between the accumulating and separating gels; 2, the large subunits; and 3, the small subunits.

3 Discussions

Differential scanning calorimetry (DSC) is a primary method to study the thermal kinetic property of proteins. By analysis of the thermal kinetic parameters for protein thermal unfolding, the cooperative interaction among domains[16], subunits[17], and molecules[18,19] can be estimated. The ratio of molar van't Hoff enthalpy to molar calorimetric enthalpy is usually indicative of the cooperativity of an investigated object. If the ratio is 1, the cooperative interaction extends to the entire protein molecule; if smaller than 1, the system-defined cooperative unit will be smaller than the unit defined by the researcher to calculate the calorimetric enthalpy; if larger than 1, the converse occurs to indicate an intermolecular interaction[20]. The DSC thermograms for some plant rubisco showed only one endothermal peak and suggested an irreversible two-state denaturation due to the high cooperativity within a rubisco molecule[6~9]. In general, hydrophobic interaction, hydrogen bond, and liganded interface are main contributions to the cooperativity of protein folding/unfolding[11]. Rubisco contains abundant aromatic residues, for example, there are 216 Phe, 232 Tyr, and 96 Trp in each molecule of spinach rubisco. The aromatic residues can provide more hydrophobic interfaces and hydrogen bonds than other residues, and so may be one of the factors that contribute to the high cooperativity during the thermal unfolding. In addition, rubisco also contains abundant cysteine residues with free sulphydryl groups (104 Cys in each molecule of spinach rubisco). Eckardt et al. reported that the heat-induced activity loss of rubisco in the absence of DTT was more sensitive than that in the presence of DTT[21]. Also Sánchez de Jiménez et al. described that the sulphydryl reactivity with 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) for native rubisco was higher than that for heat-inactivated rubisco[22]. Furthermore, the mutation of C172S in the large subunit decreased the thermal stability of the green alga rubisco[23]. As such, a question is whether the formation of disulfide bonds can contribute to the cooperativity during thermal unfolding of rubisco.

To answer this question, circular dichroism (CD), fluorescence, differential scanning calorimetry (DSC), SDS electrophoresis, and activity assays were performed on the thermal unfolding of spinach rubisco in the absence or presence of DTT. The results for CD spectra (Fig. 1) demonstrate that the secondary structure did not change from 25 to 50 ℃ and changed slightly from 50 to 60 ℃. However the results for fluorescence (Fig. 2) suggest that the tertiary and/or quaternary structure changed prior to (25 ~ 45 ℃) and faster than (45 ~ 60 ℃) the secondary structure. In addition, from about 35 to 45 ℃ was a conformational constancy. The DSC thermo-

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gram in the absence of DTT shows that the transition of rubisco (Fig. 3(a)) only corresponds to the stage of sharp conformational change (60~65 ℃, Figs. 1 and 2). The results for fluorescence in the presence of DTT demonstrate that the molecular conformation changed much faster than in the absence of DTT, and the conformational constancy disappeared. The DSC thermogram in the presence of DTT (Fig. 3(b)) shows that a slow liquidation process occurred from 45 to 60 ℃ although the main endotherm peak was primarily unchanged. In addition, when the temperature for heating rubisco was between 25 and 45 ℃ where both the second structure and the tertiary or quaternary structure were unchanged, the activity of rubisco heated either in the absence or in the presence of DTT restored completely. However, when the temperature exceeded 45 ℃ where the conformation changed increasingly with the increased temperature, the activity loss rate of the heated rubisco in the presence of DTT was much higher than that in the absence of DTT (Fig. 4). All the results above suggest that during the tertiary and/or quaternary structural alteration the dimensional positions of domains and/or subunits may provide the probability for the formation of sulfide bonds in the absence of DTT. On the contrary, the formation of sulfide bonds may be significantly disturbed in the presence of DTT. The results of SDS electrophoresis for heat-treated rubisco in the absence or presence of DTT also support the above notion and further suggest that the formation of disulfide bonds may occur mainly among the large subunits (Fig. 5(a), (b)).

Combining our results with other reports[21~23], we can draw the conclusions. (i) The disulfide bonds may be formed as early as in the stage of the tertiary and/or quaternary structural change. (ii) DTT may significantly affect the formation of disulfide bonds during thermal unfolding of rubisco. (iii) The formation of disulfide bonds in subunits and/or domains in the course of the tertiary and/or quaternary structural change may increase the thermal stability and the thermal unfolding cooperativity of rubisco.

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