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Dissociation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) observed by capillary electrophoresis

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A capillary electrophoresis method was developed to observe the dissociation of Rubisco, which is a hexadecamer enzyme with a molecular weight of *ca*. 560 kDa. In contrast to the normal separation mode in capillary electrophoresis, a minimum constant pressure together with a constant electric voltage was used to perform the separation of Rubisco. Based on this new separation mode, the conformational change during the dissociation was also observed *in vivo* by capillary electrophoresis. The equilibrium conditions of the dissociation were determined and the dynamics of the dissociation were studied in detail. It was found that the dissociation of Rubisco was completed in 4 min with the use of 0.4 mM sodium dodecyl sulfate. During the observation of dissociation, the metastable state of the conformation of Rubisco oligomer was observed by capillary electrophoresis. Moreover, the stability of Rubisco under different conditions was examined, and it was demonstrated that freeze–thawing could result in dissociation.

Introduction

The study of the dissociation and conformational changes of proteins has been a major topic in biochemistry for a long time. Traditional methods were polyacrylamide gel electrophoresis (PAGE) and UV spectrophotometry. However, they are labor intensive, show low sensitivity and high sample consumption and, most importantly, they cannot detect the dynamics of dissociation. As a rapid, sensitive and accurate method, capillary electrophoresis is now widely applied in various analyses of proteins.[1–3] Guzman used capillary electrophoresis to study protein stability in pharmaceutical formulations,[4] Liu *et al*.[5] determined the interaction between proteins and drugs with affinity capillary electrophoresis and Pahler *et al*.[6] achieved the quantitative determination of globulin in rat-kidney cytosol by capillary electrophoresis. All these applications demonstrate that capillary electrophoresis is a powerful tool for the research of proteins.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) is a key enzyme that catalyzes both photosynthesis and photorespiration in plants.[7,8] The study of its dissociation is very important, and Jiang *et al*. reported the dissociation of Rubisco by guanidine hydrochloride,[9] but the denaturation by guanidinium chloride or urea led to the inactivation of the enzyme.[10–14] Here, we developed a new separation mode and used it to detect the dissociation of Rubisco. In order to avoid the effect of a denaturing reagent on the secondary structure of the enzyme, sodium dodecyl sulfate (SDS) was applied to denature Rubisco. In addition, the stability of Rubisco was studied in detail and the metastable state of the conformation of Rubisco oligomer was observed. The dynamics of dissociation and the observation of the metastable state of a protein conformation have been first studied by capillary electrophoresis.

2. Experimental

All the reagents were purchased from Sigma (St. Louis, MO, USA).

2.1. Purification of Rubisco

Rubisco was purified from rice (*Oryza sativa*) according to Guzman.[4]

2.2 Procedure for the dissociation of Rubisco

Native Rubisco (0.386 mg ml^{-1}) was dissociated in a series of solutions containing SDS at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 mM), 50 mM Tris–HCl buffer (pH 7.8), 1 mM EDTA and 1 mM dithiothreitol (DTT) at 25 °C for 30 min.

2.3. SDS-PAGE of Rubisco

Native Rubisco was reacted with SDS solutions (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) containing 50 mM Tris–HCl buffer (pH 7.8), 1 mM EDTA and 1 mM DTT at 25 °C for 30 min. A 20 μl sample was then separated on 7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue.

2.4. Capillary electrophoresis conditions

The experiments were performed with a Beckman (Fullerton, CA, USA) P/ACE MDQ system. A fused-silica capillary (60 cm

× 100 μm, 50 cm to the detector) was purchased from Beckman. The temperature of the sample vial was 4 °C and that of the capillary was 20 °C. The running buffer was 40 mM Tris–HCl (pH 7.8). Prior to each run, the capillary was rinsed with the running buffer for 5 min at 137 920 Pa. The sample was injected into the capillary by 3448 Pa pressure for 15 s. A constant voltage (15 kV) combined with a constant pressure (3448 Pa) was applied to separate Rubisco, and the inlet end of the capillary was the anode. The wavelength of the UV detector was 280 nm.

3. Results and discussion

3.1. Effect of the separation mode on the determination of Rubisco

The isoelectric point of Rubisco was *ca*. 6, hence the surface of Rubisco should be negatively charged in Tris buffer of pH 7.0. First, a constant voltage (20 kV) with normal polarity was applied to separate Rubisco, but no peak appeared in the electropherogram. Considering the high molecular weight of Rubisco, a constant pressure (3448 Pa) was applied to the inlet end of the capillary during the separation, and then the peaks appeared (Fig. 1). With the increase in the injection volume, peak 1 increased gradually, whereas the other peaks did not show significant change. Therefore, peak 1 was identified as the peak of Rubisco.

The effect of the injection time, the separation pressure and the separation voltage on the determination of Rubisco were studied in detail. The following separations were all carried out under the optimum conditions.

3.2. Effect of the buffer system on the determination of Rubisco

Separations performed with three kinds of buffer systems were compared, namely borate, phosphate and Tris buffer (all at 100 mM). The borate buffer did not separate Rubisco at all. The current of phosphate buffer was up to 120 μA whereas that of Tris buffer was only 26 μA. To avoid Joule heating, Tris buffer was selected for the separation of Rubisco.

The average migration time of Rubisco in Tris buffer at different pH values (6.0, 7.0, 7.8 and 8.8) was 58.5 s (RSD 1.17%). This indicates that the pH has no obvious influence on the separation of Rubisco.

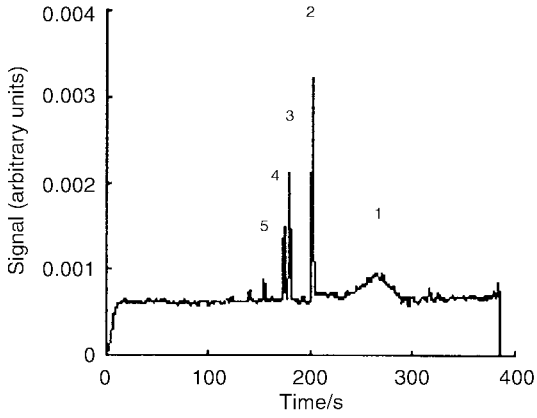

Fig. 1 Effect of the separation mode on the determination of Rubisco. 20 kV + 3448 Pa, 100 mM Tris–HCl buffer (pH 7.8); Rubisco (0.386 mg ml^{-1}) was injected by 3448 Pa for 15 min. The fused-silica capillary was 60 cm × 100 μm and the detector wavelength was 280 nm. Peaks: 1, Rubisco; 2–5, impurities.

The effect of Tris buffer concentration was also studied, and because the pH of the sample buffer was 7.8, 40 mM Tris buffer of pH 7.8 was finally adopted.

3.3 Effect of SDS concentration on the dissociation

Fig. 2 shows the electropherogram of Rubisco denatured by SDS at different concentrations. When Rubisco was not denatured [Fig. 2(A)], only the peak of Rubisco and a peak of a small subunit appeared. This demonstrated that a small subunit exists in Rubisco even under normal conditions. With increase in SDS concentration, the peak of the oligomer appeared and gradually increased (Fig. 3). There was no significant difference between the peak area of the oligomer denatured by 0.4 and 0.5 mM SDS, which indicated that the dissociation had reached equilibrium. No peak appeared in the electropherogram when only SDS was separated under the same conditions.

Fig. 4 shows the PAGE of the dissociation, and it also demonstrates that dissociation occurred when the concentration of SDS was 0.4 mM. However, only the peak of Rubisco and its small subunit appeared and the oligomer of Rubisco could not be detected clearly.

3.4. Dynamics of dissociation

The determinations discussed above all refer to dissociation *in vitro*. Here, the dissociation of Rubisco was detected *in vivo*. In the above denaturing reactions, the volume ratio of Rubisco (0.386 mg ml^{-1}) to SDS (10 mM) was 25 : 1, and they were injected under the same pressure. Here, native Rubisco (0.386 mg ml^{-1}) was first injected by 3448 Pa for 15 s, and SDS

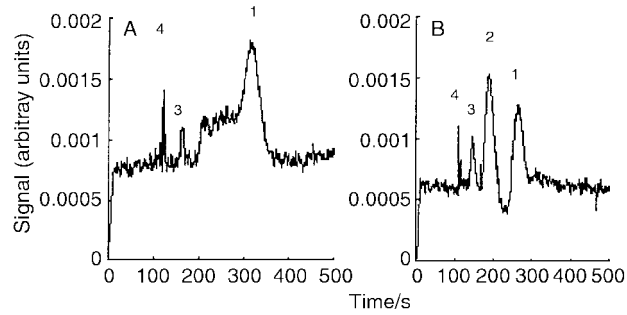

Fig. 2 Electropherogram of Rubisco denatured by SDS at 25 °C for 30 min (A) no SDS and (B) 0.3 mM SDS. The sample was injected by 3448 Pa for 15 s, and separated at 15 kV + 3448 Pa in 40 mM Tris–HCl buffer (pH 7.8). The fused-silica capillary was 60 cm × 100 μm and the detector wavelength was 280 nm. Pealis: 1, Rubisco; 2, oligomer; 3, small subunit; 4, impurity.

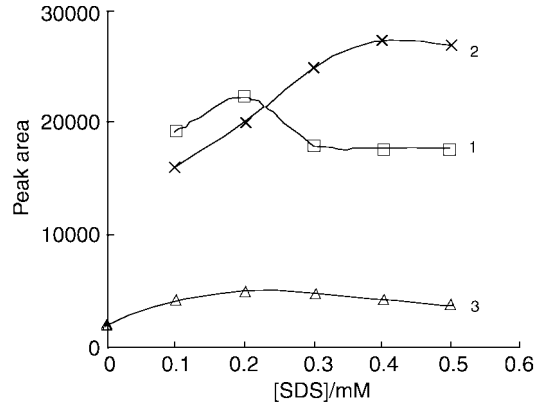

Fig. 3 Effect of SDS concentration on the dissociation of Rubisco. Pealis: 1, Rubisco; 2, oligomer; 3, small subunit. Conditions as in Fig. 2.

(10 mM) was then injected by 689 Pa for 3 s. Because the molecular weight of SDS was far lower than that of Rubisco, SDS caught up Rubisco immediately. The function of **wait** in the P/ACE MDQ plays the key role in the study of dynamics. **Wait** means that, after the injection, the sample stays in the capillary for a while before the separation. We set **wait** from 0 to 10 min, and found that the concentration of oligomer increased significantly at first and then became stable after 4 min (Fig. 5). This revealed that the dissociation reached equilibrium at 4 min. The metastable state of oligomer (indicated as 2′ in Fig. 6) was also observed during the first period of dissociation, which meant that a conformation change of the oligomer occurred during the dissociation.

3.5. Stability of Rubisco

The effect of saving conditions on the stability of Rubisco is shown in Fig. 7. After being kept at 28 °C for 24 h, no dissociation of native Rubisco occurred, but after 15 d dissociation occurred and almost reached equilibrium [Fig. 7(A)]. Further, freeze–thawing also could lead to the dissociation of Rubisco. After being kept at −20 °C for 24 h, native Rubisco dissociated, and the percentage of dissociation was higher than the above [Fig. 7(B)]. After being kept at −20 °C for 15 d, the dissociation reached equilibrium completely [Fig. 7(C)].

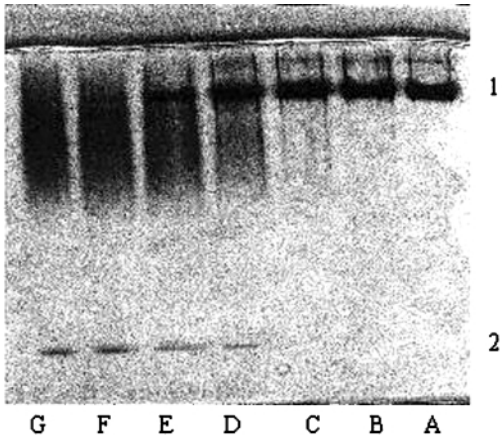

Fig. 4 PAGE of Rubisco denatured by SDS at different concentrations. Rubisco (0.386 mg ml^{-1}) was denatured by SDS at different concentrations (A, 0; B, 0.1; C, 0.2; D, 0.4; E, 0.6; F, 0.8; G,1.0 mM) at 25 °C for 30 min. 1, Rubisco; 2, small subunit. Other conditions as in Section 2.3.

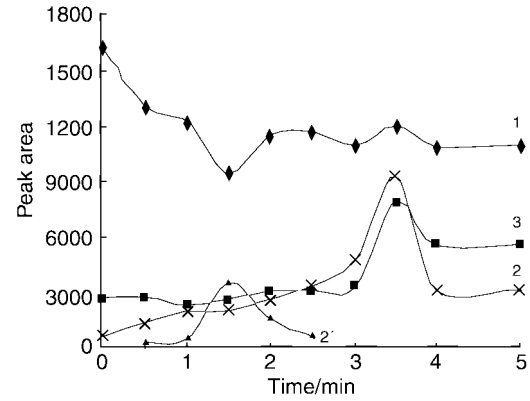

Fig. 5 Electropherogram of Rubisco after different period of dissociation. Rubisco (0.386 mg ml^{-1}) was injected by 3448 Pa for 15 s, and SDS (10 mM) was then injected by 689 Pa for 3 s. The mixture was kept in the capillary at 25 °C. Separation was carried out at 15 kV + 3448 Pa in 40 mM Tris–HCl buffer (pH 7.8). The fused-silica capillary was 60 cm × 100 μm, and the detector wavelength was 280 nm. 1, Rubisco; 2′, the metastable state of the oligomer; 2, oligomer; 3, small subunit.

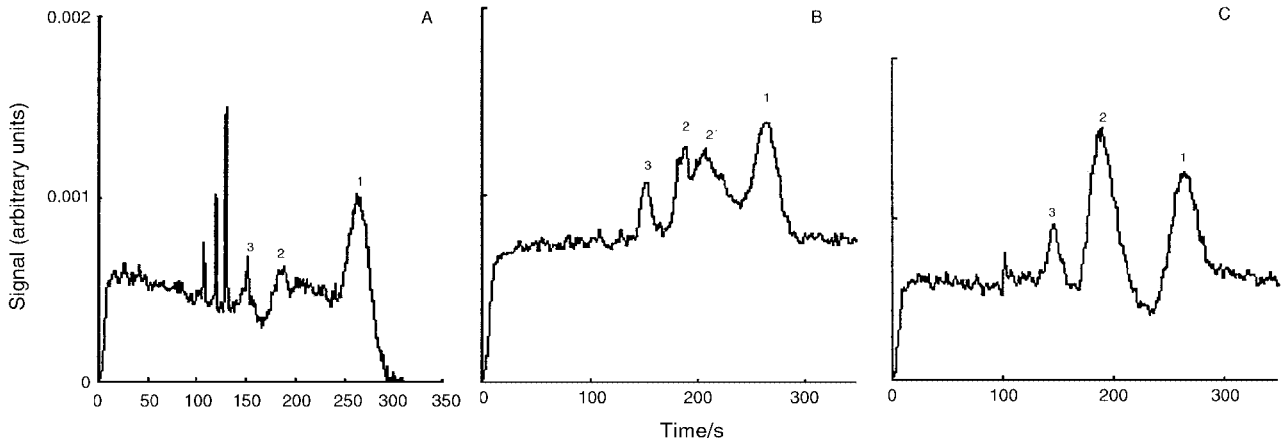

Fig. 6 Concentration change of Rubisco and its oligomer during different periods of dissociation, A, 0 min, B, 2.0 min and C, 4.0 min. Pealis: 1, Rubisco; 2′, the metastable state of oligomer; 2, oligomer; 3, small subunit. Other conditions as in Fig. 5.

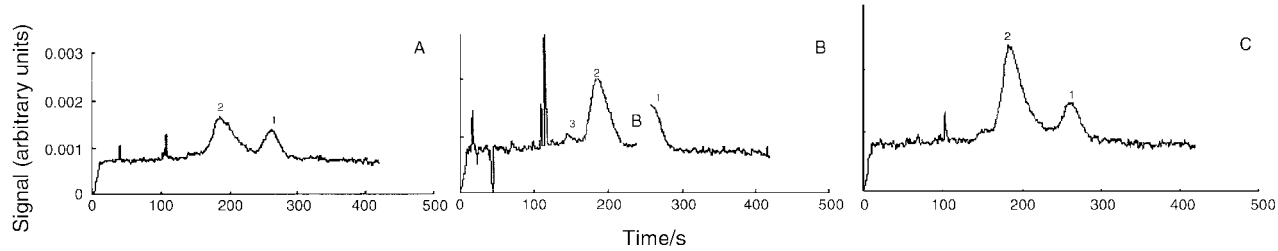

Fig. 7 Effect of the saving conditions on the stability of Rubisco. (A) Rubisco was kept at 28 °C for 15 d; (B) Rubisco was frozen for 24 h; (C) Rubisco was frozen for 15 d. Rubisco (0.386 mg ml^{-1}) was injected by 3448 Pa for 15 s; other separation conditions as in Fig. 2. Pealis: 1, Rubisco; 2, oligomer.

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