Biochemistry (Moscow), Vol. 67, No. 8, 2002, pp. 940-944. Translated from Biokhimiya, Vol. 67, No. 8, 2002, pp. 1130-1136.

Inactivation and Dissociation of Rice Ribulose-1,5-bisphosphate Carboxylase/Oxygenase during Denaturation by Sodium Dodecyl Sulfate

W. Lu[1], S. Li[2], G.-F. Li[2], Y.-D. Gong[2], N.-M. Zhao[2], R.-X. Zhang[1], and H.-M. Zhou[2]*

[1]Department of Agronomy, Nanjing Agricultural University, Nanjing 210095, China
[2]Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China; fax: 8610-62772245; E-mail: zhm-dbs@mail.tsinghua.edu.cn

Received August 9, 2001
Revision received September 19, 2001

Abstract—Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme in photosynthesis and photorespiration. The inactivation and subsequent conformational changes and dissociation of rice Rubisco by SDS have been studied. At low SDS concentrations (0.4 mM), Rubisco completely lost its carboxylase activity and most of its sulfhydryl groups became exposed. Dissociation of small subunits and significant conformational changes occurred at higher SDS concentrations. Increasing SDS concentrations caused only slight changes in CD spectrum, indicating no significant effect of SDS on the secondary structure of the enzyme. The results prove that the active site of Rubisco is more fragile to denaturants than the protein as a whole. The results also suggest that small subunits are more liable to SDS denaturation and thus dissociate first, while the more hydrophobic large subunits remain complexed. The naturally existing hydrophobic surface of Rubisco may be an important factor in the interaction of Rubisco with other macromolecules.

Key words: Rubisco, rice, inactivation, dissociation, sodium dodecyl sulfate

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) plays an essential role in CO_2 fixation during photosynthesis and photorespiration in plants [1]. It catalyzes the initial reaction in the Calvin cycle, the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate. The physiological role of Rubisco and its regulatory factors have been well discussed and documented [2-4]. Rubisco is composed of eight large subunits and eight small subunits and have a molecular weight from 520 to 560 kD in higher plants. To manifest its full activity, the enzyme should be preincubated at high concentrations of Mg^{2+} and CO_2 *in vitro* or with activase at physiological concentrations of Mg^{2+} and CO_2 *in vivo* [5]. Many investigations have analyzed the conformational changes occurring when the enzyme binds its substrate RuBP or is activated to its reactive (enzyme–carbamate–magnesium) complex (ECM) [6-8].

Studies on the inactivation and unfolding of enzymes have mainly considered monomeric or dimeric enzymes and seldom polymeric ones [9-11]. The unfolding of the hexadecameric Rubisco has rarely been studied [12, 13]. The present investigation characterizes the inactivation and dissociation of rice Rubisco in SDS solutions, which cause unfolding of some enzymes [14, 15]. The results show that the enzyme looses its carboxylase activity before detectable dissociation occurs. Comparison of the inactivation and conformational changes suggests that the active sites of this polymeric enzyme display more conformational flexibility than the enzyme molecule as a whole.

MATERIALS AND METHODS

Chemicals. 3-Phosphoglyceric phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, RuBP, SDS, and 1-anilino-8-naphthalenesulfonate (ANS) were purchased from Sigma (USA). Dithiothreitol (DTT) was a product of Promega (USA). 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) was from Serva (Germany). Other reagents were local products of analytical purity.

Purification of Rubisco from rice. The purification of Rubisco was described in [16]. Purified Rubisco was pooled

Abbreviations: ANS) 1-anilino-8-naphthalenesulfonate; DTNB) 5,5′-dithio-bis(2-nitrobenzoic acid); DTT) dithiothreitol; Rubisco) ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP) ribulose-1,5-bisphosphate; SDS) sodium dodecyl sulfate.

* To whom correspondence should be addressed.

0006-2979/02/6708-0940$27.00 ©2002 MAIK "Nauka/Interperiodica"

and judged to be homogeneous on the basis of SDS-PAGE and native PAGE. The carboxylase activity was 1.5 μmol CO_2/min per mg, and the A_{280} to A_{260} absorbance ratio was 1.9. All purification steps were carried out at 4°C.

Assay of Rubisco carboxylase activity. The carboxylase activity was determined spectrophotometrically at 25°C in assay medium containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$, 5 mM DTT, 70 mM $NaHCO_3$, 10 mM ATP, 0.4 mM NADH, 0.4 mM RuBP, 3-phosphoglyceric phosphokinase (8 U/ml), and glyceraldehyde-3-phosphate dehydrogenase (5 U/ml). Rubisco was preincubated at 25°C for 30 min with 10 mM $MgCl_2$ and 70 mM $NaHCO_3$ for full activation before the assay. Aliquots of the activated Rubisco were incubated at various SDS concentrations for 5 min and then mixed with the assay medium not containing RuBP. The reaction was initiated by the addition of RuBP, and the activity was estimated from the decrease of A_{340} [17]. The enzyme concentration was measured using Coomassie Brilliant Blue G-250 dye with BSA as the standard protein [18].

Determination of the reactive sulfhydryl groups. The number of DTNB-reactive sulfhydryl groups was determined spectrophotometrically at 25°C by measuring the increase in absorbance at 412 nm, with the molar extinction coefficient $\varepsilon = 13{,}600\ M^{-1}\cdot cm^{-1}$ [19]. After DTNB was added, the mixture was incubated for at least 1 h.

Gel electrophoresis. Native gel electrophoresis was carried out for 1 h in gels consisting of 4% polyacrylamide for the stacking gel and 7.5% polyacrylamide for the separating gel at a constant voltage of 160 V using a Bio-Rad Mini-PROTEAN® (USA) cell. The gels were stained with Coomassie Brilliant Blue R-250. Rubisco was preincubated with SDS for at least 30 min.

Conformational change measurements. Fluorescence emission spectra were measured using a Hitachi 850 (Japan) spectrofluorimeter at 25°C. The excitation wavelength was 280 nm. The ANS fluorescence emission spectra employed excitation at 380 nm. The molar ratio of ANS to Rubisco was 125 : 1. CD spectra were recorded on a Jasco 500C (Japan) spectropolarimeter at 25°C. The cell path length was 0.2 cm, the sensitivity was 100 mdeg, and each scan was repeated 20 times. Ultraviolet difference spectra were measured with a Perkin-Elmer Lambda Biospectrophotometer (USA). The composition of the solution in the reference cuvette was identical except that no enzyme was added.

All measurements were carried out in 50 mM Tris-HCl buffer (pH 8.0) at 25°C.

RESULTS

Conformational changes of Rubisco during denaturation in SDS solutions. The intrinsic fluorescence emission spectra of Rubisco at different SDS concentrations are shown in Fig. 1. It can be seen that increasing SDS concentration decreased the magnitude of the fluorescence emission spectra without any observed blue or red shift. The effect reached its maximum at 3.6 mM SDS. By contrast, SDS had little effect on the intensity of the fluorescence emission of the model compound N-acetyl-L-tryptophan [15].

Figure 2 shows the ultraviolet difference spectra of Rubisco denatured at different SDS concentrations under similar conditions. The difference spectra showed three negative peaks, at 281, 287, and 295 nm, which reached their maximum values at 4.0 mM SDS.

Secondary structure changes of Rubisco during SDS denaturation were studied using the far ultraviolet CD spectrum (Fig. 3). Used as a control, curve *7* shows the CD spectrum of the enzyme in 7 M guanidine hydrochloride solution, in which enzyme molecules are fully unfolded or disordered. The nearly similar CD spectra indicate that at the concentrations used SDS does not appreciably affect the secondary structure of rice Rubisco. All spectra showed a broad range of negative

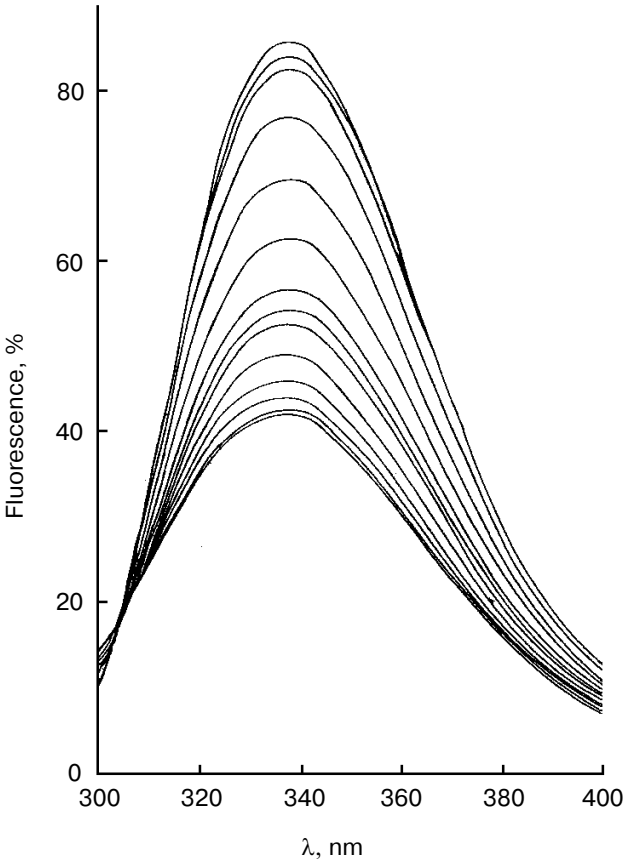

Fig. 1. Fluorescence emission spectra of rice Rubisco in the presence of SDS in 50 mM Tris-HCl buffer (pH 7.8). Enzyme concentration was 0.3 μM. The spectra were recorded after incubation for 5 min with SDS. The final SDS concentrations for curves from top to bottom were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0, 2.4, 2.8, 3.2, and 3.6 mM, respectively.

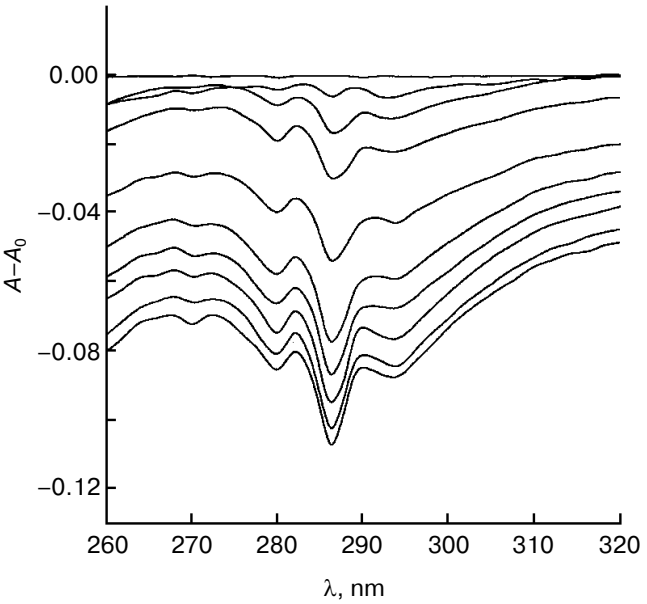

Fig. 2. Ultraviolet difference spectra of rice Rubisco at various SDS concentrations. Experimental conditions were as for Fig. 1 except that the enzyme concentration was 0.6 μM. The final SDS concentrations for curves from top to bottom were 0, 0.2, 0.6, 0.8, 1.0, 1.6, 2.0, 2.8, 3.6, and 4.0 mM, respectively.

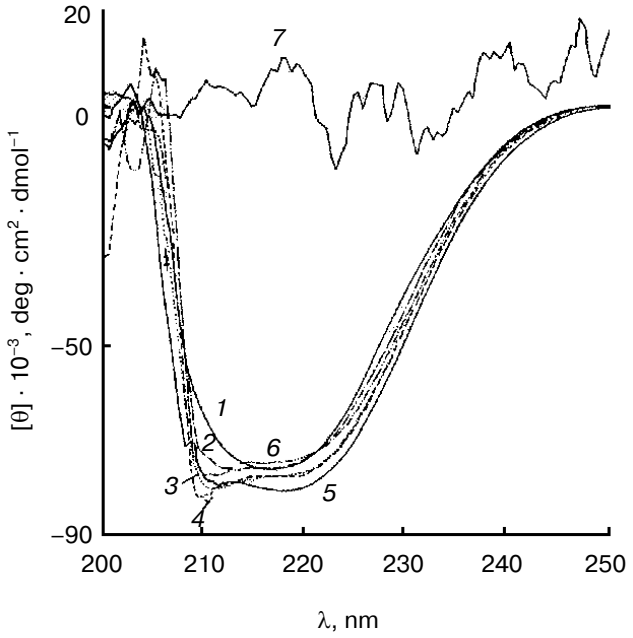

Fig. 3. CD spectra of rice Rubisco at various SDS concentrations. Experimental conditions were as for Fig. 1 except that the enzyme concentration was 0.1 μM. SDS concentrations for curves *1* to *6* were 0, 0.8, 1.6, 2.4, 3.8, and 6.2 mM, respectively. Curve *7* was for Rubisco denatured by 7 M guanidine hydrochloride.

ellipticity from about 210 to 220 nm, which is very consistent with previous research [20].

8-Anilino-1-naphthalenesulfonate (ANS) does not fluoresce in aqueous solutions but exhibits intense fluorescence when it binds to hydrophobic surfaces on proteins. In this work, ANS binding to Rubisco was investigated in the presence of SDS (Fig. 4). According to our previous study, SDS does not affect ANS fluorescence in the absence of enzyme. Therefore, the effect of SDS on ANS in the absence of enzyme could be ignored. Upon ANS binding to Rubisco, the maximum emission peak shifted from 520 to 480 nm in a biphasic manner. At an SDS concentration of 0.04 mM, ANS fluorescence reached its maximum, with a peak at 472 nm. Increasing SDS concentrations caused the fluorescence to decrease to a minimum value at SDS concentration of 3 mM. This suggests that low concentrations of SDS first cause

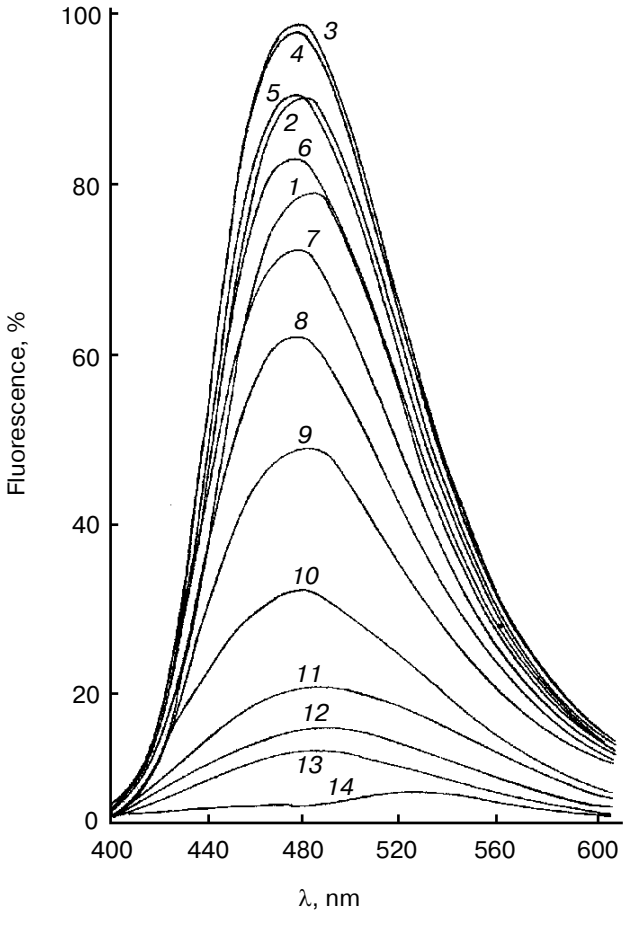

Fig. 4. ANS binding characteristics of rice Rubisco in the presence of SDS. Experimental conditions were as for Fig. 1 except that the enzyme concentration was 0.24 μM. SDS concentrations for curves *1* to *13* were 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.14, 0.2, 0.3, 0.6, 1, 2, and 3 mM, respectively. Curve *14* was for ANS in the absence of Rubisco.

Number of exposed sulfhydryl groups of rice Rubisco in various SDS solutions

SDS concentration, mM	0	0.2	0.4	0.6	0.8	1.2	3	4
Number of exposed SH groups	29.9	48.7	72.6	84.6	86.4	88.2	89.2	89.2

Note: Rubisco was dialyzed overnight to remove DTT before assay. The enzyme concentration was 0.16 μM. The molar ratio of DTNB to Rubisco was 6250 : 1.

Rubisco to form a loosened hydrophobic core. The fluorescence then decreases due to either SDS competition with ANS for the hydrophobic regions of Rubisco or the disruption of the hydrophobic surface by SDS.

DTNB is thought to react only with exposed protein SH groups since DTNB is a rather large molecule. There are 30 sulfhydryl groups on the surface of the Rubisco molecule in the absence of SDS. Increasing the SDS concentration exposed more sulfhydryl groups. A maximum of 89 sulfhydryl groups were exposed by SDS (3 M) denaturation in this work (table). The result is in good agreement with earlier reports that Rubisco has a total of 96 sulfhydryl groups per molecule [21].

Comparison of inactivation and conformational changes of Rubisco. The enzyme solutions were incubated with different SDS concentrations for 5 min before determination of the remaining carboxylase activity. The inactivation and conformational changes of rice Rubisco during SDS denaturation are compared in Fig. 5. At low concentrations, about 0.16 mM, Rubisco lost most of its carboxylase activity with all of its activity lost at 0.4 mM. However, no obvious conformational changes were observed in the enzyme molecule at these SDS concentrations. Further increases in the SDS concentration caused the enzyme to finally unfold. The exposure of sulfhydryl groups was also observed at much lower SDS concentrations than the conformational changes of the enzyme molecule, as indicated by fluorescence emission and ultraviolet difference spectra. This suggests that the SH groups may have an important role in the carboxylase activity.

Dissociation of Rubisco in SDS solutions. Figure 6 illustrates dissociation of Rubisco at varied SDS concentration, as monitored by native polyacrylamide gel electrophoresis. At the SDS concentrations higher than 0.4 mM, a diffuse band together with a clear small molecular weight band appeared below the holoenzyme band. With increasing SDS concentrations, the diffuse band became heavier and wider. We believe that 0.4 mM SDS caused detectable depletion of small subunits from Rubisco. The diffuse band probably consists of the complexes that lost only part of the small subunits. Increased SDS concentrations caused more small subunits to be

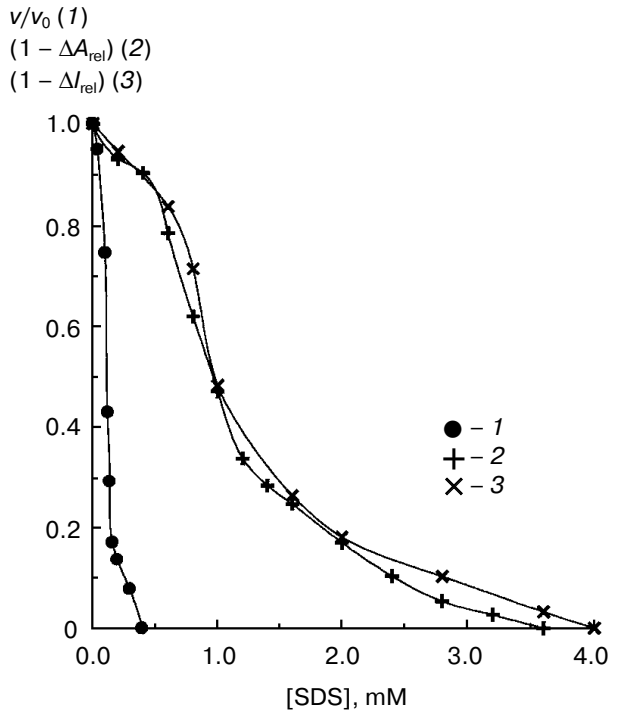

Fig. 5. Comparison of inactivation and conformational changes of rice Rubisco in the presence of SDS. Curves represent: *1*) remaining carboxylase activity (v/v_0); *2*) relative changes of difference absorbance at 287 nm ($1 - \Delta A_{rel}$); *3*) relative changes of intrinsic fluorescence intensity at 337 nm ($1 - \Delta I_{rel}$).

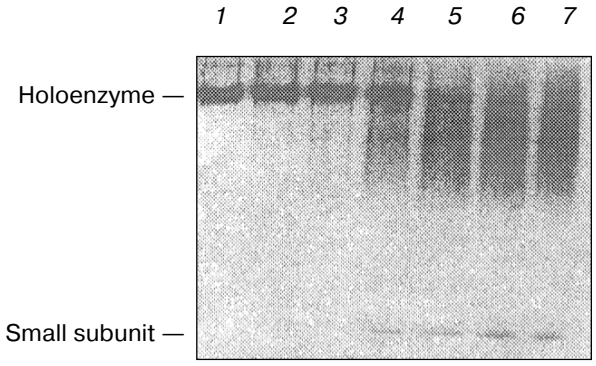

Fig. 6. Native gel electrophoresis of rice Rubisco preincubated at various SDS concentrations. The SDS concentrations were 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM for the lanes from *1* to *7*.

released. At the same time, the large subunits began to dissociate and merge into the diffuse band.

DISCUSSION

It is well known that the activity of enzymes is strongly dependent on their conformational integrity. Previously, it was reported that during denaturation of a number of enzymes by guanidine hydrochloride or urea, inactivation occurs before noticeable conformational changes of the enzyme molecule as a whole can be detected. Therefore, Tsou suggested that enzyme active sites may display more conformational flexibility than the enzyme molecules as a whole [22]. The suggestion has been widely proven for monomeric or dimeric enzymes but has seldom been investigated for polymeric enzymes as well as membrane proteins [15, 22, 23]. It has been observed that some Rubisco is bound to chloroplast thylakoid membranes by hydrophobic interactions but the physiological significance of this observation is unknown [24, 25]. Therefore, the precise relationship between inactivation and unfolding of this enzyme is of interest. The present results show that much lower concentrations of SDS are required to bring about inactivation of the carboxylase activity than are required to produce conformational changes of the enzyme molecules. The results from PAGE showed that the hexadecameric enzyme does not dissociate significantly in the presence of 0.2 mM SDS even though the enzyme is inactivated. The possibility that inactivation is due to dissociation can be excluded by PAGE. These results confirmed the flexibility of the active sites of enzymes that have some membrane protein properties, which is an important complement to Tsou's suggestion.

According to our data, small subunits began to dissociate from Rubisco at an SDS concentrations of 0.4 mM when all the carboxylase activity was lost. We suggest that increasing SDS concentrations cause the hydrophilic small subunits to dissociate from the whole enzyme molecule with the remaining hydrophobic large subunits making a loose hydrophobic core.

The observation that the addition of ANS to rice Rubisco solution results in a large enhancement of the fluorescence intensity indicates that in the native state Rubisco has some hydrophobic regions on the enzyme surface. It has been reported that a small part of Rubisco binds to the chloroplast thylakoid membrane surface while most Rubisco functions in the stroma [25, 26]. It is also interesting that low concentrations of SDS (0-0.1 mM) are required to cause the ANS fluorescence of Rubisco to increase. A reasonable explanation is that low concentrations of SDS cause Rubisco to form a loosened hydrophobic core. The naturally existing hydrophobic surface of Rubisco and its changes in different physiological conditions may provide the mechanisms for interactions between Rubisco and other macromolecules such as phospholipids in chloroplasts.

This work was supported by Chinese State Key Basic Research and Development Plan (grant G1998010100) and National Key Basic Research Specific Funds (grant No. 1999075607).

REFERENCES

1. Jensen, R. G., and Bahr, J. T. (1977) *Annu. Rev. Plant Physiol.*, **28**, 379-399.
2. Miziorko, H. M., and Lorimer, G. H. (1983) *Annu. Rev. Biochem.*, **52**, 507-535.
3. Portis, A. R. (1992) *Annu. Rev. Plant Physiol.*, **43**, 415-437.
4. Jordan, D. B., and Chollet, R. (1983) *J. Biol. Chem.*, **258**, 13751-13758.
5. Portis, A. R. (1990) *Biochim. Biophys. Acta*, **1015**, 15-28.
6. Schloss, J. V., and Hartmann, F. C. (1977) *Biochem. Biophys. Res. Commun.*, **77**, 230-251.
7. Chollet, R., and Anderson, L. L. (1977) *Biochim. Biophys. Acta*, **482**, 228-237.
8. Kwok, S. Y., and Wildman, S. G. (1974) *Arch. Biochem. Biophys.*, **161**, 354-359.
9. Bai, J. H., Xu, D., Wang, H. R., Zheng, S. Y., and Zhou, H. M. (1999) *Biochim. Biophys. Acta*, **1430**, 39-45.
10. Zhou, H. M., and Tsou, C. L. (1986) *Biochim. Biophys. Acta*, **869**, 69-74.
11. Yang, Y., and Zhou, H. M. (1998) *Biochim. Biophys. Acta*, **1388**, 190-198.
12. Voordouw, G., van der Vies, S. M., and Bouwmeister, P. P. (1984) *Eur. J. Biochem.*, **141**, 313-318.
13. Jiang, R. F., Wang, Z. X., and Xu, G. J. (1997) *Biochim. Biophys. Acta*, **1343**, 95-101.
14. Shi, Y., Luo, W., Tian, W. X., Zhang, T., and Zhou, H. M. (1998) *Int. J. Biochem. Cell Biol.*, **30**, 1319-1330.
15. Wang, Z. F., Huang, M. Q., Zou, X. M., and Zhou, H. M. (1995) *Biochim. Biophys. Acta*, **1251**, 109-114.
16. Liao, Y. G., and Li, L. R. (1991) *Acta Phytophysiologica Sinica*, **17**, 183-191.
17. Lorimer, G. H., Andrews, T. J., and Tolbert, N. E. (1975) *Meth. Enzymol.*, **42**, 484-487.
18. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-261.
19. Ellman, G. L. (1959) *Arch. Biochem. Biophys.*, **82**, 70-77.
20. Saskia, M., Paul, V. V., and Anthony, A. G. (1992) *Biochemistry*, **31**, 3635-3644.
21. Sugiyama, T., and Akazawa, T. (1967) *J. Biochem.*, **62**, 474-482.
22. Tsou, C. L. (1993) *Science*, **262**, 380-381.
23. Zhou, H. M., Zhang, X. H., Yin, Y., and Tsou, C. L. (1993) *Biochem. J.*, **291**, 103-107.
24. McNeil, P. H., and Walker, D. A. (1981) *Arch. Biochem. Biophys.*, **210**, 184-188.
25. Makino, A., and Osmond, B. (1991) *Photosynthesis Res.*, **29**, 79-85.
26. Suss, K. H., Arkona, C., and Manteuffel, R. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5514-5518.