Partial Proteolytic Digestion of PCMB-Treated Spinach Leaf Fraction-I Protein in Relation to Ribulose-1,5-Diphosphate Carboxylase Activity*

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Received June 27, 1967

Current research in this laboratory on the fraction-I protein has been focussed on the structural and catalytic role of the SH-groups in the ribulose-1,5-diphosphate (RuDP) carboxylase activity (1-3). Full restoration of the original enzyme activity of the p-chloromercuribenzoate (PCMB)-inactivated enzyme by cysteine addition was accompanied by the structural reconstitution of protein molecule demonstrable by high resolution electron microscopy (3). This finding tends to support the concept that the dissociation-association equilibrium may exist in the oligomeric subunit organization of the protein. Since this is analogous to the case of several other regulatory enzymes (4-6), it suggests a possible operation of regulatory mechanism in the RuDP carboxylase reaction by the subunit conformation change. However, so far we have been unable to characterize subunits in a transition state of the protein denaturation by physico-chemical techniques (1,2). The only detectable change induced in the protein structure was the production of diffuse protein bands on a polyacrylamide gel

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* This research is supported in part by a research grant of USPHS No. 10792-01.

electrophoregram. It will be also noted that the subsequent cleavage of the PCMB-treated protein with sodium dodecyl sulfate (SDS) did not cause any change in the mobility pattern of the "degradomers", compared with those produced by the direct treatment of protein with SDS. There is thus a possibility that another mechanism might control the conformation change of the mercurial-treated protein. In attempts to examine the possible "loosening" or "unfolding" of the protein molecule, we have tested the method of selective proteolytic digestion, such as that employed for hexokinase (7), aldolase (8,9), and alkaline phosphatase (10). All these enzymes have been shown to consist of subunits, and PCMB treatment was proved to produce the

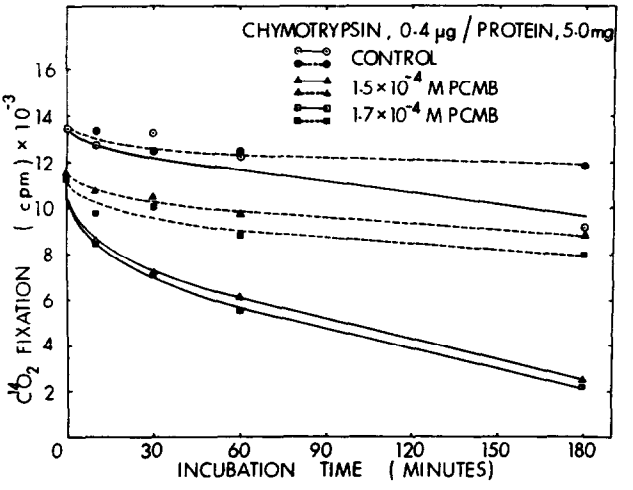

Fig. 1. Effect of Chymotryptic Digestion of PCMB-Treated Fraction-I Protein on Ribulose-1,5-Diphosphate Carboxylase Activity

To 1.8 ml (5.0 mg) of purified spinach leaf fraction-I protein (1,2) was added 0.2 ml PCMB solution of two different concentrations as indicated in the figure. In a control run, 0.2 ml of 0.1 M glycylglycine buffer (pH 7.8) was added. After incubation at 25°C for 60 minutes, 0.1 ml aliquot of the reactant was withdrawn for the enzyme assay at zero time. To examine the effect of proteolytic hydrolysis, 0.1 ml of 0.0002% α-chymotrypsin dissolved in 0.05 M Tris buffer (pH 7.8) was added to 0.9 ml of the treated protein samples and incubation continued for 10, 30, 60 and 180 minutes respectively (straight lines). As a control, 0.05 M Tris buffer (pH 7.8) was replaced (dotted lines). At each time interval, to determine the RuDP carboxylase activity 0.1 ml aliquot was added to the reaction mixture of following composition (in µmoles): Tris buffer (pH 7.8), 50; RuDP, 0.35; $MgCl_2$, 5.0; $NaHC^{14}O_3$, 25.0 (2 µc); in a total volume of 0.5 ml. Reaction was for 10 minutes at 25°C and stopped by adding 0.5 ml of acetic acid; 0.2 ml aliquot was used for the radioactivity measurement of the fixed CO_2 (1,2).

unstable enzyme form, with concomitant enhancement of the proteolytic hydrolysis. This communication concerns with the effect of two different types of proteolytic enzymes, chymotrypsin and Nagarse, on the PCMB-treated spinach leaf fraction-I protein, as measured by the changes in the RuDP carboxylase activity of this protein.

We first examined the effect of chymotrypsin, whose specific reaction pattern has been well established. Results portrayed in Fig. 1 show that the PCMB-treated protein was markedly susceptible to the chymotryptic digestion (1/12,500) in a concomitant loss of RuDP carboxylase activity, while native protein was quite resistant to the proteolysis. However, chymotrypsin of higher concentration (1/1,000) exhibited a rapid loss of the enzyme activity, which was further accerelated by the PCMB-treatment.

We next proceeded to carry out an analogous experiment using Nagarse, an alkaline bacterial protease. This enzyme is known to exhibit a rather broad specificity of the protein hydrolysis. As can be seen in Fig. 2, the carboxylase activity of the native fraction-I protein was not affected by

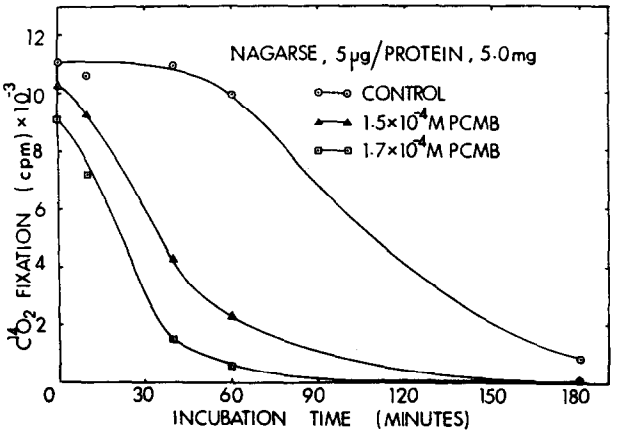

Fig. 2. Effect of Nagarse Digestion of PCMB-Treated Fraction-I Protein on Ribulose-1,5-Diphosphate Carboxylase Activity

Five mg of purified fraction-I protein was treated with PCMB at two different concentrations in the same way as that shown in Fig. 1, and 0.05 ml of 0.01 % Nagarse dissolved in 0.05 M Tris buffer (pH 7.8) was added to 1.8 ml aliquot of the incubated protein samples. After incubation at 25°C for 10, 30, 60 and 180 minutes respectively, 0.1 ml aliquot was taken up to determine the RuDP carboxylase activity using the standard reaction mixture as described in Fig. 1.

the Nagarse treatment (1/1,000) up to 60 minutes; at 180 minutes, however, less than 10% of the original enzyme activity remained. The Nagarse data thus suggests that after several bonds have been split by the proteolytic enzyme, without much loss of activity, then others become more susceptible. On the other hand, a very sharp decline of the enzyme activity occurred with PCMB treatment at the two different concentrations. Nearly complete loss of the enzyme activity occurred with the protease by 60 minutes treatment. By increasing the concentration of Nagarse 5 fold (1/200), the decline of RuDP carboxylase activity was still more drastic.

It is evident that splitting of the polypeptide chain of the fraction-I protein molecule caused the loss of the carboxylase activity, which was not restored by addition of cysteine. However, when the PCMB-inactivated

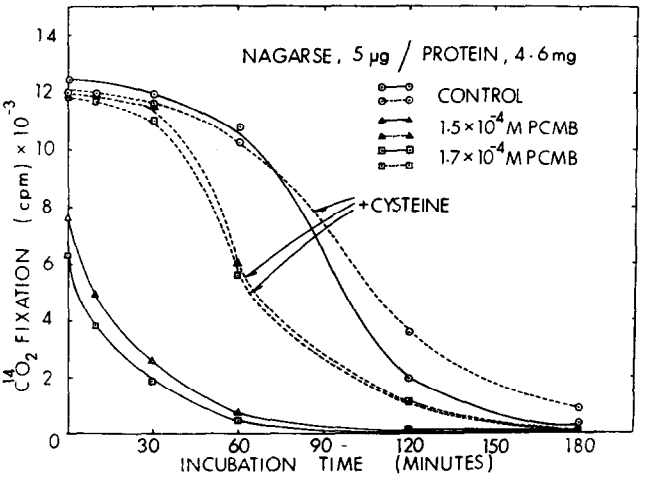

Fig. 3. Nagarse Digestion of Cysteine-Reconstituted Protein from PCMB-Inactivated Fraction-I Protein and its Effect on Ribulose-1,5-Diphosphate Carboxylase Activity

Purified fraction-I protein (4.6 mg/1.8 ml) was treated with 0.2 ml of PCMB at two different concentrations. After the incubation at 25°C for 60 minutes, 0.2 ml of either 0.05 M Tris buffer (pH 7.8) (straight lines) or 0.6 M cysteine (dotted lines) was added an incubation continued for another 30 minutes at 25°C. To determine the RuDP-carboxylase activity at zero time, 0.1 ml aliquot of the reactant was withdrawn. Simultaneously 0.05 ml of 0.01% Nagarse dissolved in 0.05 M Tris buffer (pH 7.8) was added to 2.0 ml of the reaction mixture. After incubation at 25°C for 10, 30, 60, 120 and 180 minutes respectively, 0.1 ml aliquot was withdrawn for the determination of RuDP carboxylase activity employing the standard reaction system as described in Figs. 1 and 2.

enzyme was restored by the addition of cysteine, it regained the resistance against the Nagarse hydrolysis in the 30 minutes incubation and lost its RuDP carboxylase activity in the 90 minutes treatment. Overall reaction patterns were remarkably similar to the one observed by the native protein (Fig. 3). Ultrastructural picture of the reconstituted protein particles resembled that of the original native protein (3), and the present experimental results further substantiate the complete reformation of the protein molecule, possibly identical with the native protein in its tertiary structure.

Essential point of the present experiments is that PCMB caused increased susceptibility of the structure of fraction-I protein to the proteolytic attack, as reflected in the RuDP carboxylase activity. Although this could be clearly shown at chymotrypsin 1/12,500, it was more remarkable at Nagarse 1/1,000, where the large decrements of activity occurred in the PCMB-treated enzyme during 40 and 80 minutes of incubation. Results thus strongly indicate that PCMB binding at the level of 30 to 34 moles/mole protein which caused 10 to 50% loss (cf. Figs. 1-3) of enzyme activity produced a loosening of the structure in the vicinity of the enzymic site which made it more susceptible to proteolytic attack.

In the light of recent experiments showing that the "loosening" of the subunit structure of fructose-1,6-diphosphatase governs the regulation of enzyme activity (11), it is an interesting problem to unveil whether or not the change of the spacial configuration of the fraction-I protein molecule is also concerned with the regulatory mechanism of CO_2-fixation reaction in chloroplasts. Further investigation on the structural organization of fraction-I protein may lead us a clue to elucidate the regulatory function of RuDP carboxylase in the photosynthetic act.

Acknowledgement

Authors wish to record their sincere thanks to Professor T. Murachi

for the gift of Nagarse and his many stimulating discussions in connection with this work.

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