Alterations in Structural Proteins from Chloroplast Membranes of Bacterially Induced Hypersensitive Tobacco Leaves

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ABSTRACT

Chloroplasts were isolated from tobacco leaf tissues, by homogenization and differential centrifugation, 20 min, 3 and 6 hr after infiltration with water or 10^8 cells/ml of *Erwinia amylovora*. Chloroplast membranes were prepared from isolated chloroplasts by sonication. Structural proteins (SP) were isolated from the membranes by extraction with acetone, solubilized with sodium dodecyl sulfate, and precipitated with ammonium sulfate. All SP preparations migrated as a single boundary during ultracentrifugation, and exhibited absorption maxima at 280 and minima at 250 nm. The SP isolated from tissues 20 min after infiltration with bacteria and from water-infiltrated tissues had similar optical density (OD), OD_{280}/OD_{250} ratios, and electrophoretic patterns. The SP isolated from leaf tissues 3 and 6 hr after bacterial infiltration had increased OD, decreased OD_{280}/OD_{250} ratios, and two new protein bands in electrophoretic profiles. All SP preparations were insoluble in water but able to bind lecithin; however, the SP isolated from tobacco leaf tissue 3 and 6 hr after infiltration with *E. amylovora* had decreased solubilities in 0.1 N NaOH and sodium dodecyl sulfate + urea solutions and decreased lecithin-binding capacity. The mole percent of nonpolar amino acids of structural protein decreased as a consequence of hypersensitive reaction. The changes in OD_{280}/OD_{250} ratios, solubility, phosphatide binding, and amino acid composition suggest that alteration of SP occurs during the development of the hypersensitive reaction.

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One of the predominant characteristics of the bacterially induced hypersensitive reaction (HR) is that plant cells in the inoculated area lose their turgor and the tissue becomes necrotic and desiccates. These symptoms suggest that a change in the permeability of host cell membranes occurs during the development of HR (19). Conductivity measurements of the bathing solutions of the leaf discs of tobacco

(9) and a resistant cultivar of pepper (4) revealed that intense electrolyte leakage occurred in the tissues when they were infiltrated with incompatible pathogenic bacteria. This loss of electrolytes from the tissues provided additional indication of permeability changes.

Direct evidence for the membrane damage of the cells in HR tissues was provided by electron micrographs (10). Tobacco leaf tissues showing the earliest signs of HR (6 hr after infiltration with 10^8 cells/ml of *E. amylovora*) revealed widespread membrane damage.

The structural changes observed included disruption of plasmalemma, tonoplast, bounding membranes of subcellular organelles, disorientation of chloroplast lamellar structure, and loss of integrity of mitochondrial cristae.

A current concept of membrane structure involves a backbone of protein to which lipid is bound chiefly through hydrophobic bonds (23). Fleischer et al. (7) were able to extract more than 95% of the mitochondrial lipid, apparently without affecting the appearance of mitochondrial membrane as judged by electron microscopy. Richardson et al. (23) suggested, therefore, that protein rather than lipid is primarily responsible for membrane integrity. The derangement of bounding membranes of chloroplasts and other subcellular organelles in HR tissue may therefore be a reflection of change in the nature of protein rather than lipid.

Theoretically, alteration of either membrane protein or lipid should alter cell permeability. We were able to detect the increase in phosphatidase activity in tobacco leaf tissues undergoing the bacterially induced HR; however, we were unable to induce HR with phosphatidase (12). The evidence to date, though not conclusive, suggests that a change in the nature of membrane protein rather than a potentiated phosphatidase activity is responsible for the effect of HR.

The protein constituent of the membrane was first isolated from beef heart mitochondria and was termed "structural protein" (SP) by Green et al. (11). This protein is water-insoluble at neutral pH but dissolves at pH above 10.5. It is devoid of enzymatic activity but has the ability to bind phosphatides. The SP of other membrane systems have subsequently been isolated from mitochondria and microsomes of beef liver, chloroplasts of spinach (14, 23), wheat, bean (20), and the alga, *Acetabularia* (8).

Burkowicz & Goodman (1) found that leachates from apple leaves, undergoing HR induced by an avirulent strain of *E. amylovora*, contained a greater amount of iron than those from water-infiltrated controls. Since most of the cellular iron is in chloroplasts (22), the significantly greater loss of iron from the HR tissues was interpreted as the result of altered permeability and integrity of chloroplast membranes. This mineral analysis and electron microscopic observation (1, 10) suggested that the chloroplast would be an excellent model to study membrane changes in tissues undergoing HR. In addition, chloroplast membranes are readily isolated in large quantity and high purity. Hence, the objectives of this study were (i) to isolate chloroplast SP from the tobacco leaves at different stages of HR-development; (ii) to compare their physicochemical properties; and (iii) to determine the nature of the change in SP that occurs during the development of HR.

MATERIALS AND METHODS.—*Bacterial cultures and plant materials.*—Bacterial suspensions of *Erwinia amylovora* washed with sterile distilled water from a 24-hr culture grown on nutrient agar fortified with 0.5% yeast extract and 1% glucose at 28 C were used as inocula. The concentration of the bacteria was adjusted to 10^8 cells/ml in a Bausch & Lomb Spectronic 20 at 525 nm.

Nicotiana tabacum L. 'Samsun NN' plants were grown in vermiculite, irrigated with Hoagland's solution in a growth chamber (day, 14 hr, 28 C; night, 10 hr, 18 C; light intensity, 16,130 lux). Plants with five to six expanded leaves were used in the experiments.

Isolation of structural proteins from chloroplasts.—Chloroplasts were isolated from tobacco leaves 20 min, 3 and 6 hr after infiltration with distilled water or 10^8 cells/ml of *E. amylovora*. Deveined leaves were homogenized in 0.05 M Tris [tris(hydroxymethyl) amino methane] buffer, pH 8.0, containing 0.5 M sucrose at ratio of 1:2 (w/v) in a Waring Blendor operated at full speed for 30 sec. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 200 *g* for 5 min to remove debris. We collected chloroplasts by centrifuging the supernatant at 1,000 *g* for 10 min, and washed with isolation medium. Isolated chloroplasts were then suspended in distilled water and ruptured by sonication for 15 sec in an 80 kc/sec sonic oscillator. Chloroplast membranes were obtained by centrifugation at 15,000 *g* for 15 min. Portions of the membranes were fixed for 4 hr in a 4% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.3 and washed overnight in a 0.1 M cacodylate solution containing 8.5% sucrose. The materials were postfixed in 1% OsO_4 in cacodylate buffer for 2 hr, then dehydrated, embedded, sectioned, stained, and examined according to the procedure of Huang & Goodman (13).

Structural proteins were isolated from the chloroplast membranes according to the method of Criddle (5) with slight modification. The membrane preparation was suspended in water and the suspension was poured slowly with vigorous stirring into acetone at −18 C at ratio of 1:9 (v/v). The precipitate was collected by centrifugation, washed with cold acetone and then with ether, and dried under vacuum. The resultant acetone powder was suspended in 0.002 M Tris buffer at pH 8.5 containing 0.035 M NaCl, 0.001 M sodium ascorbate and $Na_2S_2O_4$ (0.02 mg/ml). Solubilization was effected by the addition of sodium dodecyl sulfate (SDS, 0.3 g/100 ml) and urea (3 g/100 ml). After 2 hr at 4 C, the solution was centrifuged at 15,000 *g* for 15 min to remove insoluble materials, mainly starch granules which were trapped within chloroplast

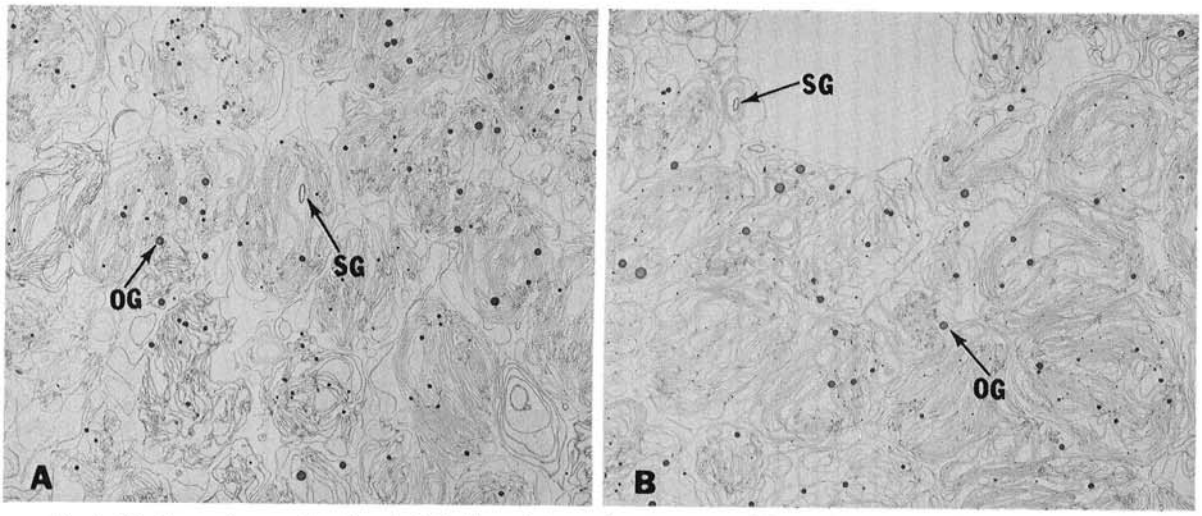

Fig. 1. Electron micrographs of sonicated chloroplast membranes prepared from tobacco tissues 6 hr after infiltration with A) water or B) 10^8 cells/ml of *Erwinia amylovora*. The membranes were used for structural protein preparations. Neither bacteria nor subcellular organelles, with the exception of osmiophilic globules (OG) and starch granules (SG), were observed (×3,100).

membranes. Saturated ammonium sulfate solution was added to the supernatant to yield a concentration of $(NH_4)_2SO_4$ of 12-16% which precipitated the structural proteins. These were repeatedly extracted with cold acetone-water (9:1, v/v) to remove the detergent and finally with ether and then dried under vacuum.

Interactions of structural proteins with phosphatide.—The phosphatide used in this study was vegetable lecithin (Mann Research Lab.). A suspension of lecithin at a concentration of 2 mg/ml was prepared by homogenization of the lecithin in 0.02 M Tris buffer at pH 7.2, and centrifuged at 10,000 *g* for 10 min to remove particles. Structural proteins dissolved in 0.1 N NaOH, pH 12.3, at a concentration of 1 mg/ml, were mixed with an equal volume of lecithin suspension. The pH of the mixture was readjusted to 12.3. After incubation at 26 C for 15 min, the pH of the mixture was brought to 7.2 with 0.1 N HCl. The insoluble protein and protein-phosphatide complex was removed by centrifugation at 3,000 *g* for 10 min and washed with water to remove free phosphatide. The pellets were assayed for phosphorus content by the procedure of Chen et al. (2).

Electrophoresis of the structural proteins.—Structural proteins were dissolved in a phenol-acetic acid-water (1:1:1,w/v) mixture at a concentration of 5 mg/ml. Gel was prepared and electrophoresis was carried out according to Takayama et al. (24). The polyacrylamide gels were stained with 1% Buffalo Black NBR in 7% acetic acid.

RESULTS.—*Electron microscopy of isolated chloroplast membranes.*—Thin sections of the sonicated chloroplasts prepared from water- and bacteria-infiltrated tissues (Fig. 1) showed that chloroplast membranes were essentially free from contamination by other subcellular organelles. Chloroplasts had already lost their bounding membranes and normal lamellar organization. Stroma components released from the chloroplasts during sonication were removed by subsequent washings, with the exception of some osmiophilic globules and starch granules in the lamellar network. Both electron micrographs and plate counts (17) showed membrane preparations to be free of bacteria.

Sedimentation and electrophoresis of the structural proteins.—Sedimentation analyses were carried out on a Spinco Model E analytical ultracentrifuge at 20 C. The medium used for sedimentation consisted of 0.1% SDS and 0.5 M urea at pH 9.5. All SP preparations migrated as a single boundary with $S_{20,w}$ values of 1.

The electrophoretic patterns of the three SP from control tissues and the SP prepared from the tissues 20 min after infiltration with bacteria were identical (Fig. 2). Five bands were observed in each gel. The electrophoretic profiles of the SP prepared from the leaf tissues 3 and 6 hr after bacterial infiltration possessed seven bands. Five of them were believed to be the same as bands that appeared in control SP. The positions of the new bands on these two gels were similar; however, the new bands were more intense in the 6-hr than the 3-hr gel.

Ultraviolet absorption of the structural proteins.—All SP preparations exhibited absorption maxima at 280 and minima at 250 nm in ultraviolet (UV) region. At a concentration of 275 μg protein/1 ml of 0.1% SDS + 0.5 M urea solution, the SP isolated from water-infiltrated leaf tissues had similar OD (Fig. 3); whereas the OD of the SP isolated from tobacco leaf tissues infiltrated with *E. amylovora* were different from one another. In addition, the OD of proteins prepared from the leaf tissues 3 and 6 hr

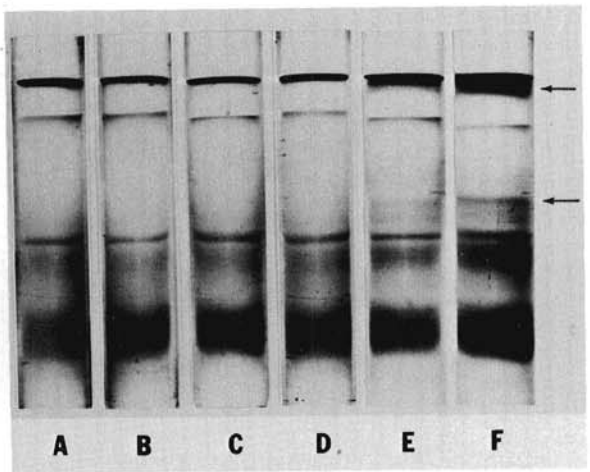

Fig. 2. Electrophoretic profiles of structural protein preparations. SP (5 mg/ml) was dissolved in phenol-acetic acid-water from which 20 μliters of protein were applied to each gel. The buffer in both upper and lower reservoirs was 10% acetic acid, with the cathode in the latter. Proteins A-C were obtained 20 min, 3 hr, and 6 hr after infiltration of leaf tissues with water and D-F at 20 min, 3 hr, and 6 hr after infiltration of tissues with bacteria. New proteins are indicated by arrows.

TABLE 1. Solubility of chloroplast membrane proteins isolated from tobacco leaf tissues infiltrated with *Erwinia amylovora* or water

Structural protein source	Time after inoculation	Solubility (mg/ml) 0.1 N NaOH	Solubility (mg/ml) 0.1% SDS[d] + 0.5 M urea
H_2O[a]	20 min	3.4[c]	8.6
H_2O	3 hr	3.5	8.6
H_2O	6 hr	3.2	8.4
Ea[b]	20 min	3.6	8.6
Ea	3 hr	3.0	6.5
Ea	6 hr	2.5	6.0

[a] Membrane structural protein from leaves infiltrated with H_2O.

[b] Membrane structural protein from leaves infiltrated with 10^8 cells/ml *E. amylovora*.

[c] Average of three structural protein preparations. Three determinations were made on each preparation.

[d] SDS = sodium dodecyl sulfate.

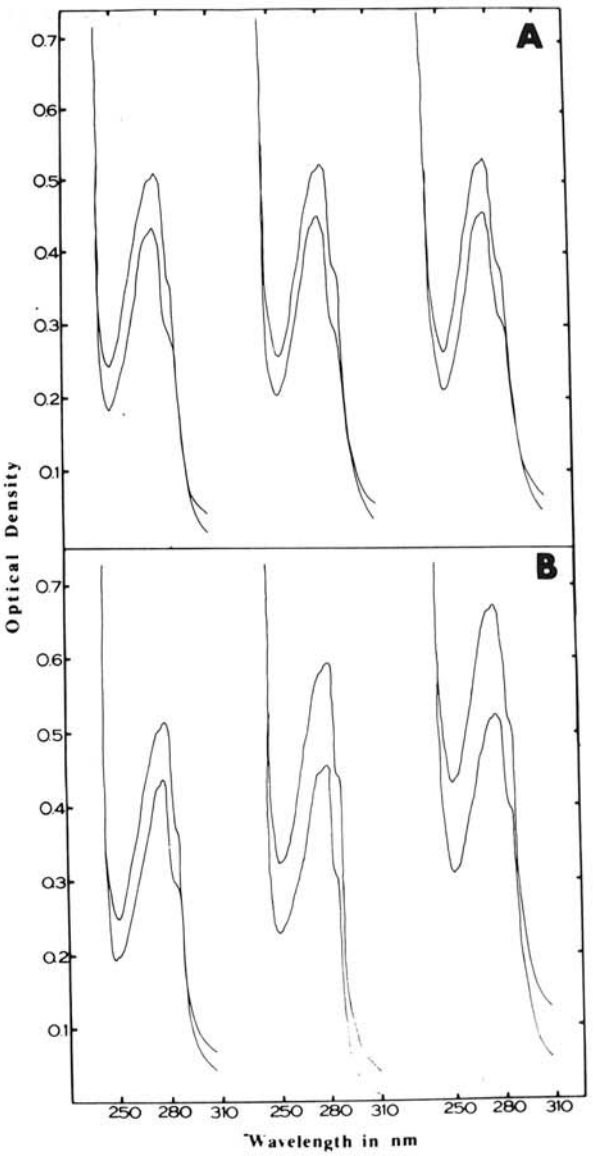

Fig. 3. Ultraviolet absorption spectra of unheated (lower lines) and heated (upper lines) structural proteins isolated from leaf tissues 20 min (left), 3 hr (center), and 6 hr (right) after infiltration with water (A) or 10^8 cells/ml of *Erwinia amylovora* (B). Proteins were dissolved in 0.1% sodium dodecyl sulfate + 0.5 M urea solution at a concentration of 275 μg/ml.

after bacterial infiltration were greater than either the water-infiltrated samples or the sample obtained 20 min after infiltration with bacteria.

All six samples were then heated at 100 C for 60 min, and the volumes of the solutions were readjusted to the ones before heating. Spectrophotometric measurements disclosed marked increases in absorption near 250 nm and less marked increase near 280 nm (Fig. 3).

Heat treatment decreased the OD_{280}/OD_{250} ratios of the three proteins prepared from the water-infiltrated tissues and the two proteins obtained from the tissues 20 min and 3 hr after infiltration with bacteria (Fig. 4). However, heat treatment of the protein isolated from the tissues 6 hr after bacterial infiltration caused no additional decrease in the OD_{280}/OD_{250} ratios.

Solubility of structural proteins.—All SP preparations were completely insoluble in water. The solubilities of SP in 0.1 N NaOH and of the 0.1% SDS + 0.5 M urea solution are given in Table 1. SP, isolated from leaf tissues 3 hr and 6 hr after infiltration with *E. amylovora*, exhibited further

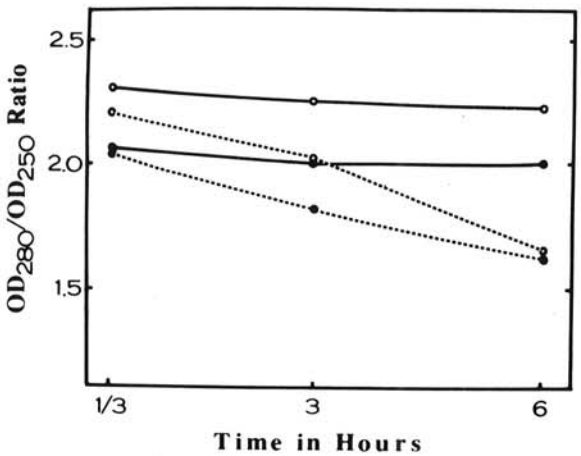

Fig. 4. The OD_{280}/OD_{250} ratios of the unheated (open circles) and heated (closed circles) isolated from tobacco leaf tissues 20 min, 3 hr, and 6 hr after infiltration with water (solid lines) or 10^8 cells/ml of *Erwinia amylovora* (dotted lines). The proteins were dissolved in 0.1% sodium dodecyl sulfate + 0.5 M urea solution at a concentration of 275 μg/ml.

TABLE 3. Mole percent of amino acids of chloroplast structural proteins isolated from tobacco leaf tissues infiltrated with *Erwinia amylovora*

Amino acid	Time after infiltration		
	20 min	3 hr	6 hr
Aspartic acid	9.6	10.6	8.9
Threonine	3.6	3.3	4.9
Serine	3.1	3.4	4.0
Glutamic acid	9.3	8.5	8.8
Proline	1.6	1.4	1.0
Glycine	11.6	11.3	9.4
Alanine	10.2	10.7	10.1
0.5 Cystine[a]	ND[b]	ND	ND
Valine	8.0	7.5	6.7
Methionine	Trace	Trace	Trace
Isoleucine	5.7	5.2	7.2
Leucine	12.2	11.4	9.5
Tyrosine	2.9	2.9	4.8
Phenylalanine	9.0	10.7	8.4
Histidine	2.4	2.9	4.3
Lysine	5.7	6.8	7.7
Arginine	5.2	3.4	4.2

[a] Cystine and cysteine were detected as cysteic acid and expressed as 0.5 cystine.
[b] ND = not detectible.

TABLE 2. Amount of phosphatide bound by chloroplast membrane proteins isolated from tobacco leaf tissues infiltrated with *Erwinia amylovora* or water

Structural protein source	Time after inoculation	μg of phosphorus/mg of protein	
		Initially	In final pellet[c]
H_2O[a]	20 min	0.8[d]	10.3
H_2O	3 hr	0.8	11.0
H_2O	6 hr	0.8	10.7
Ea[b]	20 min	0.8	10.6
Ea	3 hr	0.9	6.5
Ea	6 hr	0.9	5.1

[a] Membrane structural protein from leaves infiltrated with H_2O.
[b] Membrane structural protein from leaves infiltrated with 10^8 cells/ml *E. amylovora*.
[c] Lecithin was incubated with protein (2:1, w/w) at pH 12.3, 26 C, for 15 min. The complex was removed by centrifugation. The pellet was washed and the amounts of protein and phosphorus were determined.
[d] Average of three structural protein preparations. Three determinations were made on each preparation.

decreased solubility in the two protein solvents.

Binding ability of structural proteins to phosphatide.—The amount of phosphatide bound to the SP as measured by the amount of phosphorus present in the protein-lipid complex is shown in Table 2. The SP isolated from water-infiltrated tobacco leaf tissues and the one isolated from leaf tissues 20 min after bacterial infiltration bound approximately the same amount of phosphatide. The SP isolated from tobacco leaf tissues 3 and 6 hr after bacterial infiltration showed reduced binding capacity. In fact, there was an additional reduction of 22% in phosphatide binding by SP obtained 6 hr after infiltration as compared with the 3-hr sample.

Amino acid compositions of the structural proteins.—Amino acid analyses were performed using a BioCal BC-200 amino acid analyzer. The proteins were hydrolyzed with 6 N HCl at 110 C. Since the 3 SP of water-infiltrated controls were essentially identical to the SP prepared from tobacco leaf tissues 20 min after bacterial infiltration, only the compositions of the SP isolated from bacteria-infiltrated tissues are listed in Table 3. The data suggest two general characteristics for these protein preparations: (i) SP contain a high percentage of nonpolar amino acids and, as a consequence, membrane proteins are considered hydrophobic in nature; and (ii) SP contain no sulfhydryl or disulfide groups. The amino acid composition of SP observed 20 min and 3 hr after bacterial infiltration was similar, differing by more than 1 mole percent for only three amino acids (lysine, phenylalanine, and arginine). The compositional change between the 20 min and 6 hr SP, however, was much greater with eight amino acids (threonine, glycine, valine, isoleucine, leucine, tyrosine, histidine, and lysine) differing by more than 1 mole percent. The mole percents of nonpolar amino acids for 20 min, 3 hr, and 6 hr SP are 61.1, 61.1, and 57.1, respectively, suggesting that nonpolar amino acids decrease following the induction period of HR.

DISCUSSION.—That the chloroplast membranes used for SP extraction were of high purity is evident in Fig. 1, wherein neither bacterial cells nor recognizable subcellular organelles were observed. SP were also free of soluble proteins, as none was detected in the water in which SP were suspended.

The presence of a single sedimenting boundary

after ultracentrifugation suggested that SP from control and HR-tissues are homogeneous. However, electrophoresis separated each protein preparation into several components (Fig. 2), indicating that SP were heterogeneous, at least with respect to charge. Similar data have been reported for chloroplast membrane proteins from spinach (14), bean, and wheat (20). The appearance of two new protein bands in the electrophoretic profiles of 3-hr and 6-hr SP prepared from bacteria-infiltrated tissues reflect configurational and charge changes that are detectable in an electrical field. These changes may be too subtle to alter sedimentation characteristics.

Spectrophotometric studies in the ultraviolet region disclosed that when the protein ovomucoid was heated it exhibited increased absorption (OD) near 250-255 and 275-280 nm, with the greatest increases occurring at the minimum absorption wavelengths. Hence, the ratio of OD_{280}/OD_{250} decreased as the protein became denatured (6). The three SP isolated from water-infiltrated (control) leaf tissues had similar OD in the unheated state. They increased their ultraviolet absorption (Fig. 3) and decreased their OD_{280}/OD_{250} ratios (Fig. 4) to approximately the same extent upon heat treatment. These observations indicate that the SP isolated from leaf tissues 20 min, 3 hr, and 6 hr after water infiltration have analogous properties with respect to ultraviolet absorption. It is also apparent that the SP of the tobacco chloroplast membranes has spectrophotometric characteristics similar to water-soluble ovomucoid in both unheated and heated states. The SP isolated from leaf tissues 20 min after bacterial infiltration was comparable in these respects to the water-infiltrated controls (Fig. 3, 4). It would appear, therefore, that 20 min after infiltration with *E. amylovora* the SP had not yet been affected. On the other hand, the SP isolated 3 hr and 6 hr after bacterial infiltration exhibited higher ultraviolet absorption values and lower OD_{280}/OD_{250} ratios, both indicative of denaturation. Of interest was the observation that, after heating, there was a further decrease in OD_{280}/OD_{250} ratio of SP prepared from the tissues 3 hr but not 6 hr after bacterial infiltration. Apparently, the 6 hr-SP had altered "fully" as a consequence of HR.

The loss of water solubility of globular proteins is generally accepted as a criterion of denaturation (15). Similarly, we have interpreted the decrease of the solubility of the SP isolated from leaf tissues 3 and 6 hr after bacterial infiltration as an indication of denaturation.

Cole et al. (3) reported that denaturation of membrane proteins could affect their ability to reassociate with lipid to form membranelike structures. The decrease in the binding capacity between lecithin and the SP isolated from leaf tissues 3 and 6 hr after bacterial infiltration is therefore considered as another indication of protein denaturation.

Denaturation of a protein is defined by Joly (15) as a modification of the secondary, tertiary, or quaternary structure of the protein molecule, and excludes covalent bond breakage. According to this definition, denaturation does not involve changes in amino acid composition. Many proteins, however, vary their amino acid composition as a consequence of denaturation (15). For example, ovomucoid, lysozyme, and deoxyribonuclease change the concentrations of 7, 5, and 5 amino acids, respectively, upon heat or irradiation treatment (6, 21). Similarly, the change in the amino acid composition of SP isolated from leaf tissues 3 and 6 hr after bacterial infiltration (Table 3) may be the result of denaturation.

Another possible interpretation of our data is that biosynthesis of chloroplast membrane proteins may be altered. For example, altered amino acid incorporation into SP may be responsible for the observed changes in electrophoretic patterns, ultraviolet absorption, solubility, and phosphatide binding. This contention is supported by the comprehensive change in amino acid composition between the 3-hr and 6-hr SP prepared from bacteria-infiltrated tissues. The minimal change in amino acid composition between 20 min and 3 hr may reflect the induction phase of HR, recently reported by Klement (18) to be about 3 hr. If, for example, the synthesis of chloroplast membrane protein is altered by the bacteria, the greatest change would manifest itself subsequent to the induction of HR, and could result in the protein changes recorded by us. Of interest is the fact that the turnover rate (half life) of mycoplasma membrane protein is 3 hr (16).

The data we have presented indicate that SP of chloroplast membranes isolated from tissues undergoing HR changes its physicochemical properties. Some of these changes are demonstrated 3 hr after bacterial infiltration; at that time, no changes in membrane permeability or ultrastructure are detected (9, 10). Whether the changes in SP are due to denaturation or altered synthesis is as yet unknown. It would appear, however, that the observed alterations in SP are responsible for the changes in membrane permeability and structural integrity observed at 6 hr (9, 10).

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