PRESENCE OF THE RAPIDLY-LABELLED 32 000-DALTON CHLOROPLAST MEMBRANE PROTEIN IN TRIAZINE RESISTANT BIOTYPES

Autar K. MATTOO*, Jonathan B. MARDER, Jonathan GRESSEL and Marvin EDELMAN
Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 6 January 1982

1. Introduction

A large number of herbicides, including triazines, inhibit photosynthesis by blocking electron transport at the reducing side of photosystem II [1–3]. The triazines are presently considered to interfere with the electron flow between Q, the primary electron acceptor of photosystem II, and the secondary quencher, B. Binding studies with radiolabelled atrazine [2-chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine] and diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] have been interpreted as indicating an overlap in their thylakoid binding sites [1,2,4,5]. On mild trypsin digestion of isolated thylakoids, photosystem II electron transport is either inhibited or becomes insensitive to diuron and atrazine, depending on the redox acceptor used [1,2,6,7]. This trypsin-effect was recently correlated with partial digestion of the rapidly-metabolized 32 000 dalton thylakoid protein of *Spirodela* [8]. Thylakoids from this organism depleted of 32 000 dalton protein in vivo have a decreased capacity for electron transport on the reducing side of photosystem II [8]. When azido-[^{14}C]atrazine was covalently attached to thylakoid proteins of *Amaranthus* by photoaffinity labelling, a 32 000 dalton polypeptide was the major radioactive band [9]. Although there are other possibilities [10], these and further [11] reports point to a 32 000–34 000 dalton polypeptide of the photosystem II complex as forming at least a portion of the atrazine receptor site.

Several studies by Arntzen and co-workers have shown that thylakoid membranes of triazine resistant biotypes, which have recently appeared in susceptible species, have a greatly reduced affinity for atrazine [2,5,12] and azido-atrazine [9]. This prompted us to investigate whether atrazine resistant plants synthesize the 32 000 dalton protein. We report here that both triazine resistant and susceptible biotypes of *Brassica campestris*, *Bromus tectorum*, *Chenopodium album* and *Solanum nigrum* actively synthesize a 32 000 dalton thylakoid protein. This protein is shown to be comparable to the rapidly-metabolized 32 000 dalton protein of *Spirodela* [13] and other plants [14] as regards partial peptide mapping and positioning within the thylakoid membrane.

2. Materials and methods

Seeds of triazine resistant and susceptible biotypes of *Brassica campestris*, *Bromus tectorum*, *Chenopodium album* and *Solanum nigrum* were kindly supplied by V. Souza-Machado, Guelph, Ontario; J. Hensley, Greensboro, North Carolina; S. Radosevich, Davis, California; and J. Gasquez, Dijon, France, respectively. Plants were grown from seeds in a green-house under normal lighting conditions. Fully grown leaves were washed thoroughly with distilled water and rinsed with sterile water. Leaf discs (15 mm diameter) were incubated for 4 h in half-strength Hutner's medium [cf. ref. 15] without sucrose under 3000 lux white light. One gram fresh weight of discs was incubated with 20 ml medium in a sterile Petri dish (9 cm). At the end of incubation, the medium was changed with 5 ml of fresh medium containing 0.4% Tween 20 and 250 μCi of [^{35}S]methionine (1000 Ci/mmol; Amersham). After labelling for 3.5 h, discs were washed with distilled water and suspended in

* Present address: Plant Hormone Laboratory, PPHI, Agricultural Research Center (West), US Department of Agriculture, Beltsville, MD 20705, USA

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

30 ml of ice-cold 0.4 M sucrose, 0.01 M Tricine–NaOH (pH 8 at 20°C) and 0.01 M NaCl. Chloroplasts were prepared as in [8]. Chlorophyll was determined in 80% (v/v) acetone extracts of thylakoid membranes [16].

Samples with equal amounts of radioactivity were fractionated on sodium dodecylsulphate (SDS) 10–20% polyacrylamide gradient gels [17]. After fixing in 7% acetic acid–20% methanol, gels were either dried directly for autoradiography or prepared for fluorography [18] and exposed on Curix RP2 X-ray films (Agfa). Limited proteolysis of polypeptides excised from dried gels was carried out on 15–20% SDS–polyacrylamide gradient gels with *Staphylococcus aureus* V8 protease (Miles) or papain (Sigma) as described in [19]. [^{35}S]Methionine-labelled chloroplast membranes were digested with trypsin (Worthington) at concentrations and times as indicated under fig.3. Other conditions of digestion with trypsin were as described in [8].

3. Results and discussion

Fig.1 shows the electrophoretic patterns of Coomassie-stained chloroplast membrane polypeptides of triazine resistant (*r*) and susceptible (*s*) biotypes of *Solanum nigrum*, *Chenopodium album*, and *Brassica campestris*. Qualitatively the stained patterns were similar for each pair of biotypes but with some differences between species. Fig.1 also shows the autoradiogram of the newly-synthesized thylakoid polypeptides. A 32 000 dalton polypeptide was the most prominent synthesis product in all the samples pulse-labelled with [^{35}S]methionine irrespective of triazine resistance or susceptibility. In samples from *B. campestris* and *C. album* the radioactive 32 000 dalton band was not associated with a Coomassie-stained band while in *S. nigrum* it was. This latter association, however, appears fortuitous as the stained band was unaffected by trypsin under conditions which produced a distinctive digestion pattern for the rapidly-labelled 32 000 dalton protein (not shown). Other labelled bands which can be identified are the high M_r-value subunit of photosystem I and the α- and β-subunits of the plastid ATPase. A 30 000 dalton chloroplast polypeptide was also rapidly labelled. It coincided with a stained band in *B. campestris* but not in the other species. We have not investigated the identities of the 30 000 dalton bands. Overall, no major difference in the in vivo synthesized protein products could be seen within any pair of the *r* and *s* biotypes.

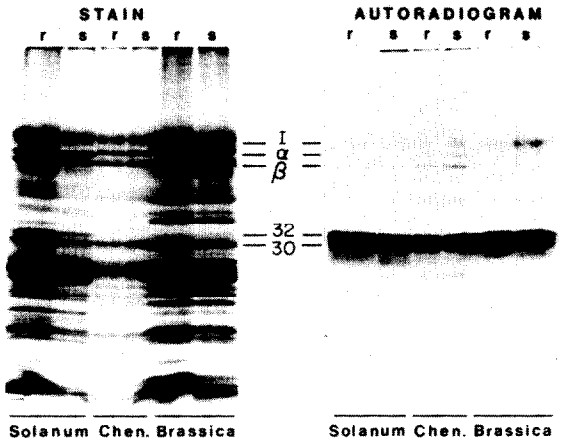

Fig.1. Stained and radiolabelled thylakoid proteins of triazine resistant (*r*) and susceptible (*s*) biotypes of *Solanum nigrum*, *Chenopodium album* and *Brassica campestris* after fractionation on an SDS–polyacrylamide gel. Conditions of labelling with [^{35}S]methionine, and electrophoresis, are described in section 2. The positions of I (a high M_r-value subunit of the photosystem I reaction center), α and β (subunits of the proton ATPase), 32 000 dalton and 30 000 dalton polypeptides are indicated.

Several chloroplast membrane polypeptides with apparent molecular masses in the range of 32 000–34 000 have been reported. Some of these are: the chloroplast-coded, rapidly-synthesized 32 000 dalton polypeptide in pea [20] and *Spirodela* [13,17], the 34 000 dalton protein located on the oxidizing side of photosystem II in *Scenedesmus* [21], the histidine-lacking 33 000 dalton polypeptide in spinach [22], the 34 500, 34 000 and two 32 000 dalton polypeptides in maize thylakoids [23] and the 33 000 dalton polypeptide in the cyanobacterium *Aphanocapsa* [26]. In view of these reports we considered it important to ascertain the identity of the 32 000 dalton proteins synthesized by the various triazine *r* and *s* plants. Partial proteolytic digestion of the 32 000 dalton polypeptides was carried out with two different proteases by the method in [19]. The patterns obtained are shown in fig.2. *S. aureus* protease and papain cleaved the 32 000 dalton polypeptides into specific patterns of fragments indistinguishable from one biotype to another. These patterns were similar to those characteristic of the rapidly-synthesized 32 000 dalton protein from *Spirodela* and other higher plants [14].

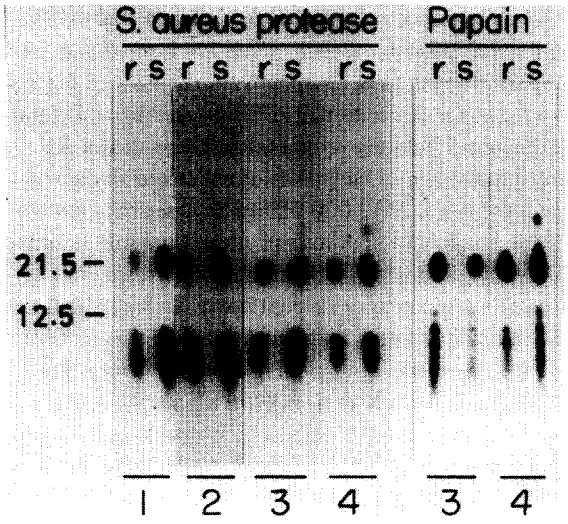

Fig.2. Partial proteolytic digestion of the 32 000 dalton protein band from triazine resistant (*r*) and susceptible (*s*) biotypes. Leaf discs were labelled with [^{35}S]methione. Membranes were isolated and fractionated by SDS–polyacrylamide gel electrophoresis. Following autoradiography the 32 000 dalton polypeptide bands were located and excised from the dried gels. The gel pieces were then treated [16], fractionated in a second 15–20% gradient polyacrylamide gel in the presence of 5 μg of *S. aureus* V8 protease or 0.05 μg papain, and fluorographed. The origins of the 32 000 dalton bands were: lane 1-*Solanum nigrum*; lane 2-*Brassica campestris*; lane 3-*Chenopodium album*; lane 4-*Bromus tectorum*. The positions of [^{14}C]methylated M_r-value standards (Amersham) are indicated [soybean trypsin inhibitor (21 500 dalton) and cytochrome *c* (12 500 dalton)].

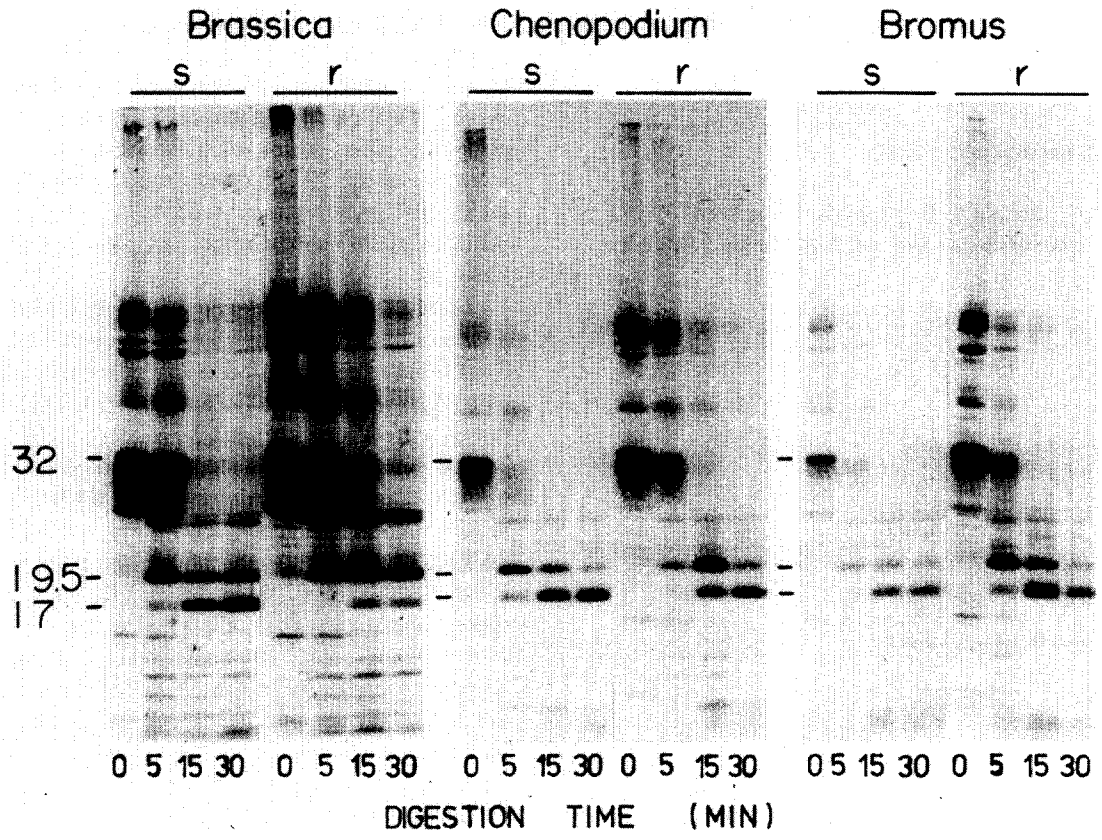

Fig.3. Distribution of rapidly labelled proteins after trypsin digestion of thylakoids from triazine susceptible (*s*) and resistant (*r*) biotypes. [^{35}S]Methionine-labelled thylakoid membranes, equivalent to 175 μg chlorophyll/ml, were digested with trypsin (Worthington) at 50 μg/ml in 10 mM Tris–HCl (pH 7.6), at 22°C for the times indicated. Trypsin digestion was stopped by a 10-fold dilution of samples in a solution of lima-bean trypsin inhibitor (2.5 mg/ml) and phenylmethylsulfonylfluoride (1 mM). All samples were prepared, electrophoresed on SDS–polyacrylamide gels and autoradiographed as in [7]. The positions of the 32 000 dalton protein and its digestion products (19 500 dalton and 17 000 dalton) are indicated.

The rapidly-synthesized 32 000 dalton chloroplast protein is known to be partially exposed at the thylakoid surface [8,23] and thus is susceptible to mild trypsin digestion [6,7]. Following enzyme application to isolated thylakoids, relatively stable membrane-associated fragments of 19 500 dalton and 17 000 dalton are produced [14]. Fig.3 shows the electrophoretic patterns of [^{35}S]methionine-labelled proteins of thylakoids from *r* and *s* biotypes treated with trypsin for different periods of time. In all cases digestion of the 32 000 dalton protein coincided with the appearance of a 19 500 dalton fragment, which on prolonged incubation was further digested to a 17 000 dalton fragment. The results of these comparisons indicate that: (1) a close similarity in structure and membrane topography exists between the newly-synthesized 32 000 dalton polypeptides of the weed species investigated here and those more fully characterized in *Spirodela*, maize and peas [8,13,14,23]; (2) the same 32 000 dalton polypeptide is synthesized by the triazine *r* and *s* biotypes.

No consistent difference in the kinetics of appearance of proteolytic products was found between the rapidly-synthesized 32 000 dalton protein of *r* and *s* biotypes. However, the 32 000 dalton polypeptides in the membranes of the *s* biotypes of *Brassica campestris* and *Chenopodium album* appeared slightly more susceptible to trypsin than their counterparts from the *r* biotypes. (Compare, for example, the relative proportions of the 19 500 dalton and 17 000 dalton fragments in fig.3 after 15 min digestion.) Thus a slightly altered conformation of this polypeptide in the chloroplast membranes of triazine *r* biotypes is possible. However, this altered situation may be independent of triazine sensitivity and occur due to non-isogenicity in the biotype pairs.

From our data it is clear that atrazine resistance is not due to a loss of expression of the gene for the 32 000 dalton protein, as synthesis of the 32 000 dalton polypeptide occurs at similar rates in both triazine *r* and *s* biotypes. Proteins of 32 000 daltons in both biotypes have a similar mobility on denaturing polyacrylamide gels and possess common cleavage sites for three different proteases. Thus, it appears that the primary structures of triazine *r* and *s* 32 000 dalton proteins is not grossly different. However, minor differences, for example, a change in a single amino acid, would not be revealed by our experiments unless they occurred fortuitously at a cleavage site. A direct answer to this possibility must therefore await sequencing of the 32 000 dalton protein or its gene. There are several alternative possibilities, among them that atrazine resistance in weed biotypes may result from a change in the microenvironment of the 32 000 dalton protein, rather than, or in addition to, a change in the 32 000 dalton protein itself. In this regard we note the difference in thylakoid lipid composition found between several resistant and susceptible biotypes [24,25]. It has also been proposed that azido-atrazine might act as a bifunctional reagent and that the 32 000 dalton polypeptide is actually located very near to the atrazine binding protein [10]. We note, however, that even in this last case the gene for the 32 000 dalton protein may carry mutations conferring herbicide resistance due to the proximity of the 32 000 dalton protein to the site of atrazine binding.

Acknowledgement

The expert technical assistance of Mrs Dina Heller is gratefully acknowledged.

References

[1] Trebst, A. and Draber, W. (1979) Adv. Pesticide Sci. 2, 223–234.
[2] Pfister, K. and Arntzen, C. J. (1979) Z. Naturforsch. 34C, 996–1009.
[3] Ashton, F. M. (1981) Mode of Action of Herbicides, Wiley, New York.
[4] Tischer, W. and Strotmann, H. (1977) Biochim. Biophys. Acta 460, 113–125.
[5] Pfister, K., Radosevich, S. and Arntzen, C. J. (1979) Plant Physiol. 64, 995–999.
[6] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[7] Regitz, G. and Ohad, I. (1976) J. Biol. Chem. 251, 247–252.
[8] Mattoo, A. K., Pick, U., Hoffman-Falk, H. and Edelman, M. (1981) Proc. Natl. Acad. Sci. USA 78, 1572–1576.
[9] Pfister, K., Steinback, K. E., Gardner, G. and Arntzen, C. J. (1981) Proc. Natl. Acad. Sci. USA 78, 981–985.
[10] Gressel, J. (1982) Plant Sci. Lett. in press.
[11] Mullet, J. E. and Arntzen, C. J. (1981) Biochim. Biophys. Acta 635, 236–248.
[12] Arntzen, C. J., Ditto, C. L. and Brewer, P. E. (1979) Proc. Natl. Acad. Sci. USA 76, 278–282.
[13] Edelman, M. and Reisfeld, A. (1980) in: Genome Organization and Expression in Plants (Leaver, C. J. ed) pp. 353–362, Plenum, New York.
[14] Hoffman-Falk, H., Mattoo, A. K., Marder, J. B. and Edelman, M. (1982) J. Biol. Chem. in press.

[15] Posner, H. B. (1976) in: Methods in Developmental Biology (Wilt, F. A. and Wessels, N. K. eds) pp. 301–317, Crowell, New York.
[16] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[17] Weinbaum, S. A., Gressel, J., Reisfeld, A. and Edelman, M. (1979) Plant Physiol. 69, 828–832.
[18] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 76, 83–88.
[19] Cleveland, D. W., Fischer, S. G., Kirschner, W. M. and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106.
[20] Blair, G. E. and Ellis, R. J. (1974) Biochim. Biophys. Acta 319, 223–234.
[21] Metz, J. and Bishop, N. I. (1980) Biochem. Biophys. Res. Commun. 94, 560–566.
[22] Kuwabara, T. and Murata, N. (1979) Biochim. Biophys. Acta 581, 228–236.
[23] Grebanier, A. E., Coen, D. M., Rich, A. and Bogorad, L. (1978) J. Cell Biol. 78, 734–746.
[24] Blein, J.-P. (1980) Physiol. Veg. 18, 703–710.
[25] Pillai, P. and St. John, J. B. (1981) Plant Physiol. 68, 585–588.
[26] Astier, C. and Joset-Espardellier, F. (1981) FEBS Lett. 129, 47–51.