Biochimica et Biophysica Acta 851 (1986) 202–208
Elsevier

BBA 42100

Herbicide and plastoquinone-binding proteins of Photosystem II reaction center complexes from the thermophilic cyanobacterium, *Synechococcus* sp.

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(Received February 4th, 1986)

Key words: Herbicide-binding protein; Photoaffinity labeling; Photosystem II; Plastoquinone-binding protein; Reaction center; (*Synechococcus* sp.)

Photoaffinity labeling of *Synechococcus* Photosystem (PS) II preparations with radioactive azido-derivatives of three herbicides and of plastoquinone was carried out to identify herbicide and plastoquinone-binding proteins. [^{14}C]Azido-atrazine and [^{14}C]azido-monuron specifically labeled the 28 kDa polypeptide of the PS II reaction center complex, which is sensitive to 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). No specific labeling of this polypeptide with azido-atrazine was found in CP2-b (PS II reaction center lacking the 40 kDa subunit) which is insensitive to DCMU. [^{3}H]Azido-dinoseb reacted with the 28 kDa polypeptide and the 47 kDa chlorophyll-carrying protein. The labeling with [^{3}H]azido-plastoquinone resulted in the incorporation of the radioactivity exclusively into the 47 kDa polypeptide. It is concluded that the 28 kDa polypeptide is the herbicide-binding protein of the cyanobacterium and that the 47 kDa polypeptide has a binding site for plastoquinone and for phenol-type herbicides.

Introduction

Photoaffinity labeling with radioactive azido-analogues of herbicides has been extensively used to identify polypeptides of the thylakoid membranes to which the herbicides specifically bind [1–7]. [^{14}C]Azido-atrazine in higher plants and green algae consistently labels an intrinsic protein in the 30–34 kDa molecular mass region, which is considered to be the Q_B-binding protein [1–3]. there have been, however, two reports which suggest that the polypeptide labeled by azido-atrazine is not the herbicide-binding protein [8,9]. Metz et al. [9] recently presented evidence that in a mutant of *Scenedesmus* azido-atrazine tags a 36 kDa polypeptide. Gressel [8] argues that azido-atrazine might not label the herbicide-binding protein, but a neighbouring protein instead. Most recently, azido-triazinone [4] and azido-monuron [7] have been introduced as two new photoaffinity labels of the 'DCMU-type'. Both unambiguously bind to an identical 34 kDa polypeptide as does azido-atrazine [4,7]. Additionally, azido-monuron also labels a 41 kDa polypeptide [7].

Another group of herbicides, the so-called 'phenol-type' herbicides have a binding site which at least partly overlaps that of the 'DCMU-type' herbicides [10]. Nevertheless, the photoaffinity labeling suggests that the receptor of the phenol-type herbicides is present in a larger polypeptide(s). Azido-dinoseb reacts, in addition to polypeptide(s)

Abbreviations: PS, Photosystem; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; azido-atrazine, 2-azido-4-(ethylamino)-6-(isopropylamino)-*S*-triazine; azido-monuron, 3-(4′-azido-3′-chlorophenyl)-1,1-dimethylurea; azido-dinoseb, 2-azido-4-nitro-6-isobutylphenol; azido-triazinone, 3-dimethylamino-4-methyl-6-(3′-azidophenyl)-1,2,4-triazin-5-one; azido-plastoquinone, 2,3-dimethyl-5-{4′[3-(4-azido-2-nitroanilino)propionoxy]-*n*-butyl}-1,4-benzoquinone; Chl, chlorophyll.

in the 30 kDa region, with polypeptides in the 40–53 kDa mass region which are chlorophyll-carrying large subunits of the PS II reaction center complex [5,6,11].

There are two, and possibly three, plastoquinone-binding sites in the PS II reaction center complex, i.e., the Q_B site, the Q_A site and, if plastoquinone, the Z site. The binding of a plastoquinone molecule at the Q_B site is considered to be inhibited by herbicides. Oettmeier et al. [12] have shown that [^{3}H]azido-plastoquinone labels a polypeptide of 32 kDa which migrates differently from the polypeptide labeled by azido-atrazine in SDS gels.

The ambiguity in the identification of the herbicide and quinone-binding proteins by means of the photoaffinity labeling would be considerably reduced by the use of purified PS II preparations with a simple and well defined subunit composition. The PS II reaction center complexes isolated from higher plants and algae are mostly insensitive to 'DCMU-type' herbicides and do not always give a specific labeling of the 30–34 kDa polypeptide with azido-atrazine [11], suggesting the absence or modification of the herbicide-binding site. Of particular interest in this respect are PS II preparations obtained from the thermophilic cyanobacterium, *Synechococcus* sp., which grows at high temperature and hence has very stable proteins or enzymes [13]. The PS II reaction center complex isolated from the cyanobacterium shows a high and DCMU-sensitive activity of 2,6-dichlorophenolindophenol photoreduction with 1,5-diphenylcarbazide as an electron donor [14]. This indicates that one of the two polypeptides of 28 and 31 kDa present in the complex is the competent herbicide-binding protein. The complex contains, besides the 28 and 31 kDa polypeptides, two chlorophyll-binding polypeptides of 47 and 40 kDa and one or two polypeptides of 9 kDa which may be the apoprotein of cytochrome *b*-559. The function of the two chlorophyll-binding polypeptides has been well established. The complex can be splitted into two complemental chlorophyll-protein complexes called CP2-b and CP2-c. CP2-b, which contains the 47 kDa polypeptide together with diminished levels of smaller polypeptides, but no 40 kDa polypeptide, shows photoreduction of Q_A and pheophytin [15] and photooxidation of P-680 [16], suggesting that the 47 kDa polypeptide carries the PS II reaction center. Another chlorophyll-protein complex containing only the 40 kDa polypeptide (CP2-c) shows no PS II photochemical activities and hence is considered to be an intrinsic antenna of the PS II complex. These PS II preparations are, therefore, particularly suitable samples for the identification of the herbicide and quinone-binding proteins.

In the present work, we have carried out photoaffinity labeling of *Synechococcus* PS II preparations with radioactive azido-derivatives of atrazine, DCMU, dinoseb and plastoquinone. The results indicate that the 28 kDa polypeptide is the herbicide-binding protein of the cyanobacterium. A large molecular weight polypeptide of 47 kDa was labeled by azido-dinoseb. It also has a binding site for azido-plastoquinone.

Materials and Methods

An oxygen-evolving PS II preparation, a PS II reaction center preparation and a PS II complex which lacks a chlorophyll-carrying 40 kDa polypeptide (CP2-b) were prepared as reported previously [15,17] from the thermophilic cyanobacterium, *Synechococcus* sp. grown at 55°C [18]. The oxygen-evolving PS II complexes were partially purified by DEAE-Toyopearl column chromatography in the presence of digitonin [19].

[^{14}C]Azido-atrazine (specific activity, 25 mCi/mmol) and [^{14}C]azido-monuron (specific activity, 59 mCi/mmol) were purchased from Amersham-Buchler, Braunschweig, F.R.G. [^{3}H]Azido-dinoseb (specific activity, 350 mCi/mmol) and [^{3}H]azido-plastoquinone (specific activity, 214 mCi/mmol) were synthesized as described recently [5,20].

For photoaffinity labeling, samples were incubated with an azido-derivative at 0°C in the dark for 5 min. Labeling with azido-atrazine and azido-monuron was carried out by illuminating the incubated samples with ultraviolet light from a mercury lamp for 15 min. Illumination for 10 min with strong white light from a 2000 W halogen lamp was used in the case of azido-dinoseb and of azido-plastoquinone [20]. After labeling, polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (11.5–15%) as reported previously [5], except that 6 M urea was present in the gels.

The gels were stained with Coomassie brilliant blue and then cut into pieces of 1 mm width. After dissolving the gel pieces, radioactivity of the gel pieces was measured in a liquid scintillation counter [5].

Results

Fig. 1 shows the radioactivity distribution of polypeptides resolved from PS II reaction center complexes labeled by [^{14}C]azido-atrazine. Among polypeptides resolved from the complexes, the 28 kDa polypeptide was associated with the highest radioactivity. The specific labeling of the 28 kDa polypeptide was also demonstrated in partially purified oxygen-evolving particles (not shown). The radioactivity found in the 47 kDa polypeptide which split into two bands under these electrophoretic conditions for unknown reason, was negligible. The 40 kDa polypeptide, the 9 kDa apoprotein of cytochrome *b*-559 and polypeptides in the 14–16 kDa region resulting from a contamination by allophycocyanin subunits were not appreciably labeled, either. The 31 kDa polypeptide was only labeled to some minor extent. In contrast, no specific binding of azido-atrazine to the 28 kDa polypeptide was observed in the CP2-b preparation in which the distribution of radioactivity appears to parallel roughly the amounts of polypeptides resolved (Fig. 2). This seems to be related to the lack of DCMU-sensitivity in the CP2-b preparation [15]. We conclude, therefore, that the 28 kDa polypeptide is the herbicide-binding protein of the *Synechococcus* PS II reaction center complexes. Satoh [2] has shown that from the two polypeptides in the 30–34 kDa region

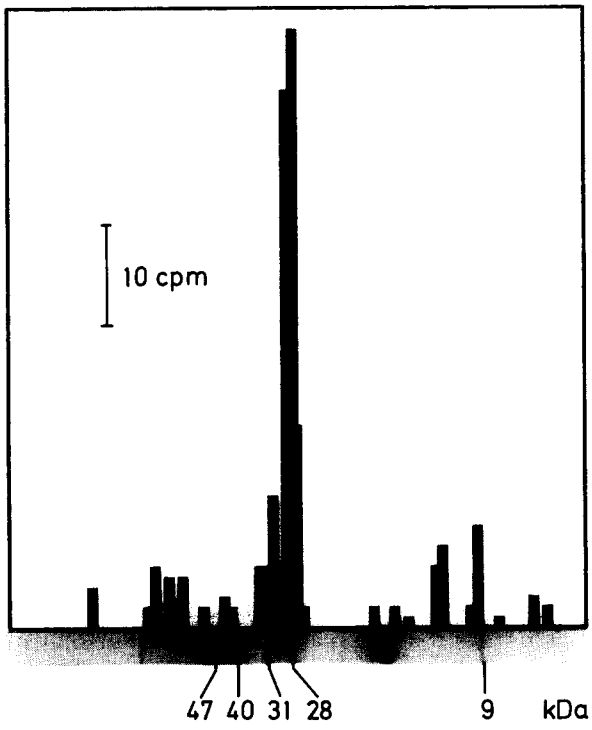

Fig. 1. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of the PS II reaction center preparation labeled with [^{14}C]azido-atrazine. [^{14}C]Azido-atrazine (0.79 mM, 0.3 μl) was added to 50 μl of the reaction center preparation (17.3 nmol/mg Chl). The chlorophyll *a* concentration was 274 μg/ml.

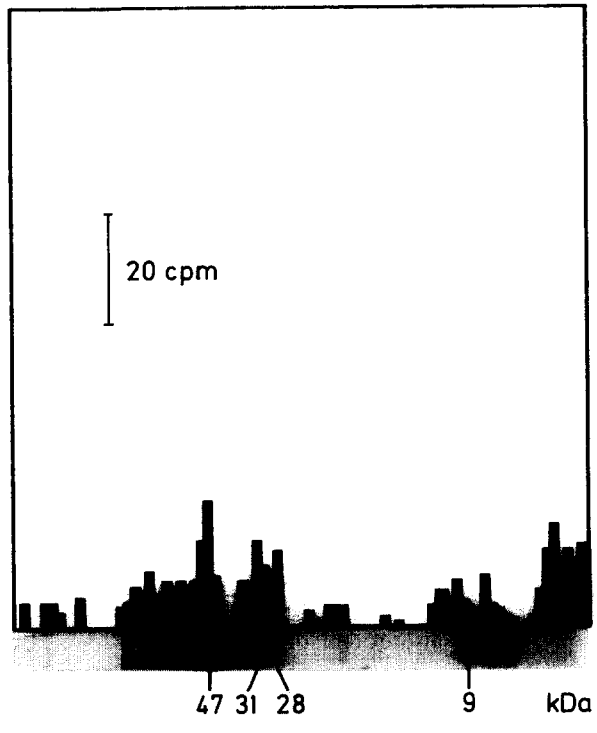

Fig. 2. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of the CP2-b preparation labeled with [^{14}C]azido-atrazine. [^{14}C]Azido-atrazine (0.79 mM, 0.3 μl) was added to 40 μl of CP2-b (13.0 nmol/mg Chl). The chlorophyll *a* concentration was 366 μg/ml.

present in the spinach PS II reaction center complex, the one with a faster electrophoretic mobility is specifically labeled by [^{14}C]azido-atrazine.

Experiments were also carried out with an azido-derivative of DCMU (Fig. 3). It should be noted that the azido-monuron used in this study differs slightly from that in Ref. 7, which is the 3′-azido-4′-chloro-isomer. The binding region of DCMU is similar to but not identical with that of atrazine because several mutants that are resistant to atrazin show an unaltered sensitivity to DCMU [21]. Moreover, the position of the azido group in azido-monuron is different from that in azido-atrazine with respect to the position of the active structural element –NH–C=X, where X is N or O [22]. Fig. 3 shows that the 28 kDa polypeptide of the PS II reaction center complex was exclusively tagged by [^{14}C]azido-monuron. Similar results were obtained with the oxygen-evolving PS II preparation (data not shown). These results reinforce our conclusion that the 28 kDa polypeptide is the herbicide-binding protein in *Synechococcus*.

Photoaffinity labeling of PS II preparations with a radioactive azido-derivative of a phenol-type herbicide, [^{3}H]azido-dinoseb, are shown in Fig. 4 and 5. Azido-dinoseb is a rather unspecific label which reacts with a number of proteins present in the thylakoid membranes [11]. We also observed that all polypeptides of *Synechococcus* oxygen-evolving preparations have affinity for azido-dinoseb, although extents of the labeling considerably varied with polypeptides (not shown). Fig. 4 shows a labeling experiment with the PS II reaction center complexes. To lower the level of unspecific binding, it is essential to incubate the samples at a high chlorophyll to azido-dinoseb

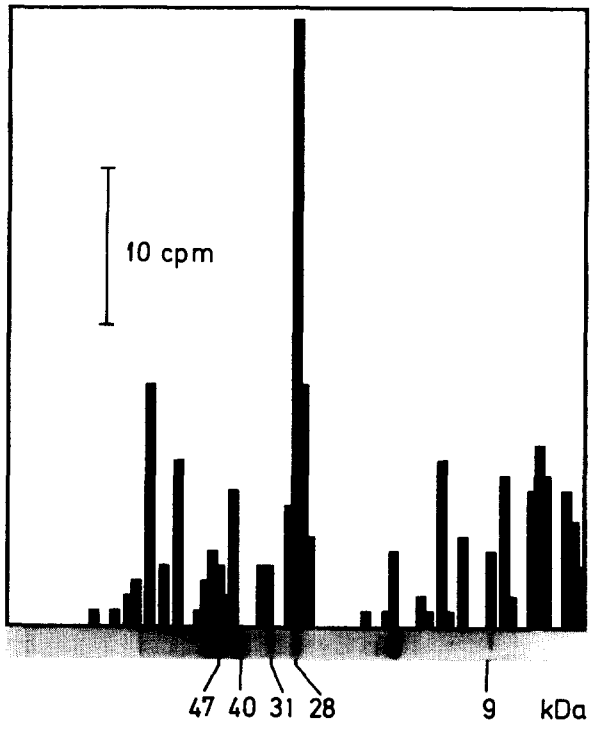

Fig. 3. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of the PS II reaction center preparation labeled with [^{14}C]azido-monuron (15 nmol/mg Chl). [^{14}C]Azido-monuron (0.5 mM, 0.45 μl) was added to 50 μl of the reaction center preparation (274 μg Chl *a*/ml).

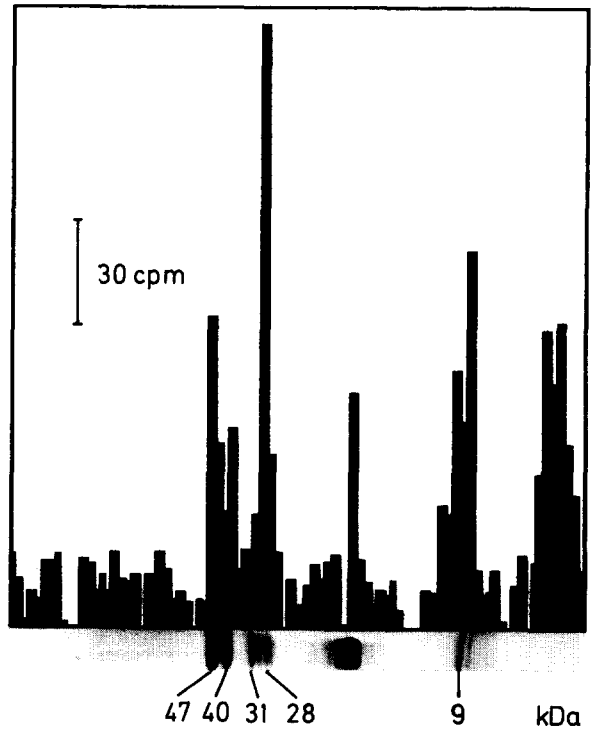

Fig. 4. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of the PS II reaction center preparation labeled with [^{3}H]azido-dinoseb. [^{3}H]Azido-dinoseb (0.26 mM, 1.0 μl) was added to 50 μl of the reaction center preparation. Chlorophyll *a* concentration was 274 μg/ml.

ratio and to keep the sample temperature exactly at 0°C during the illumination with strong white light. The good resolution of polypeptides and radioactivity bands allow us to identify labeled polypeptide and furthermore to estimate extents of the labeling of individual polypeptides. The highest radioactivity was incorporated into the 28 kDa herbicide binding protein, followed by the 9 and 47 kDa polypeptides. It is to be mentioned here that the labeling of the 28 and 47 kDa polypeptides was highly reproducible, while the 9 kDa polypeptide was occasionally not associated with a significant radioactivity. The labeling of the 47 kDa polypeptide was clearly demonstrated with CP2-b preparation used which largely lacked polypeptides in the 30 kDa region (Fig. 5).

Finally, we carried out the photoaffinity labeling of PS II preparations with [^{3}H]azido-plastoquinone. The previous experiments have shown that the plastoquinone derivative labels most of polypeptides present in the thylakoid membranes, presumably by binding unspecifically to hydrophobic regions of proteins [12]. It is, therefore, remarkable that the azido-plastoquinone tagged only two of the six major polypeptide bands resolved from partially purified oxygen-evolving PS II complexes (fig. 6). It is seen that the 47 kDa polypeptide was strongly labeled. The 40 kDa polypeptide and the 35 kDa extrinsic polypeptide have migrated closely but the radioactivity band corresponds better to the 40 kDa band. No radioactivity was detected in the 28 kDa herbicide-binding protein and most of the radioactivity migrated with SDS micelles on the gel front.

Experiments were extended to CP2-b and the results illustrated in Fig. 7 show that only the 47 kDa polypeptide was associated with the radioactivity band. These results strongly suggest that the

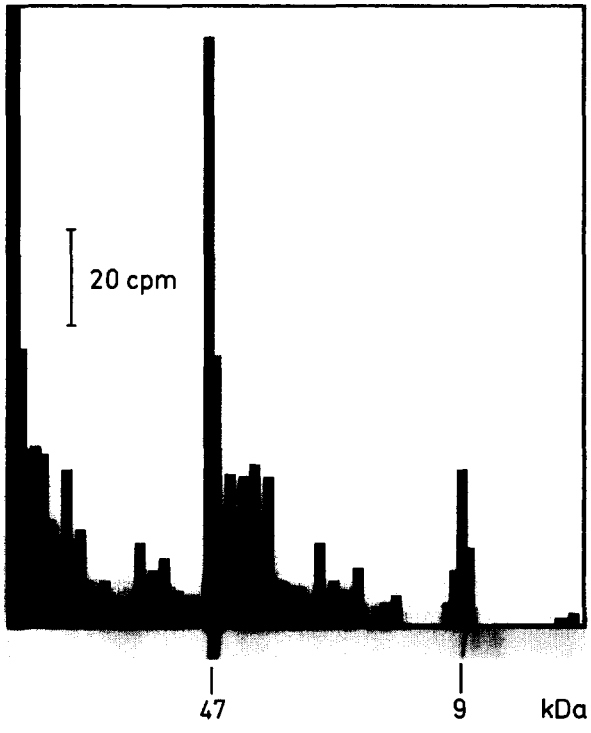

Fig. 5. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of a CP2-b preparation labeled with [^{3}H]azido-dinoseb. [^{3}H]Azido-dinoseb (0.26 mM, 1.0 μl) was added to 40 μl of CP2-b. Chlorophyll *a* concentration was 366 μg/ml.

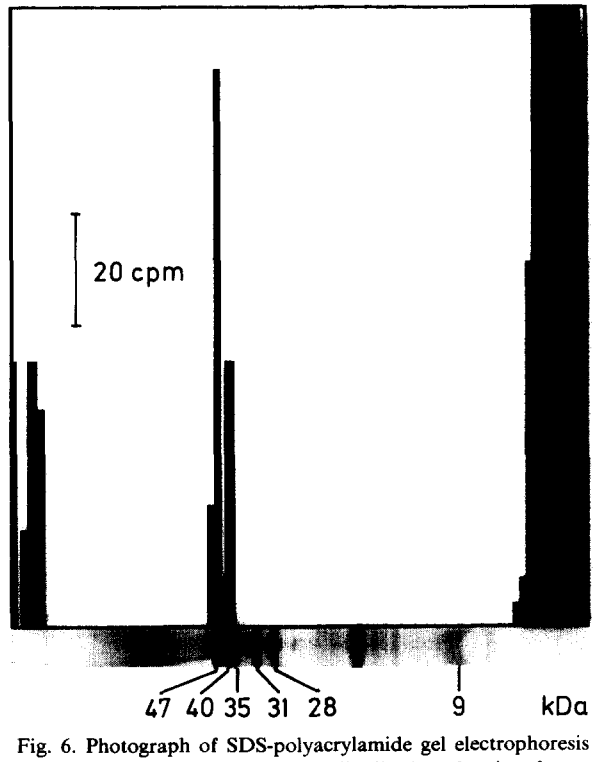

Fig. 6. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of partially purified oxygen-evolving PS II complexes labeled with [^{3}H]azido-plastoquinone (7.7 nmol/mg chl). [^{3}H]Azido-plastoquinone (0.514 mM, 0.2 μl) was added to 30 μl of oxygen-evolving PS II complexes (445 μg Chl *a*/ml).

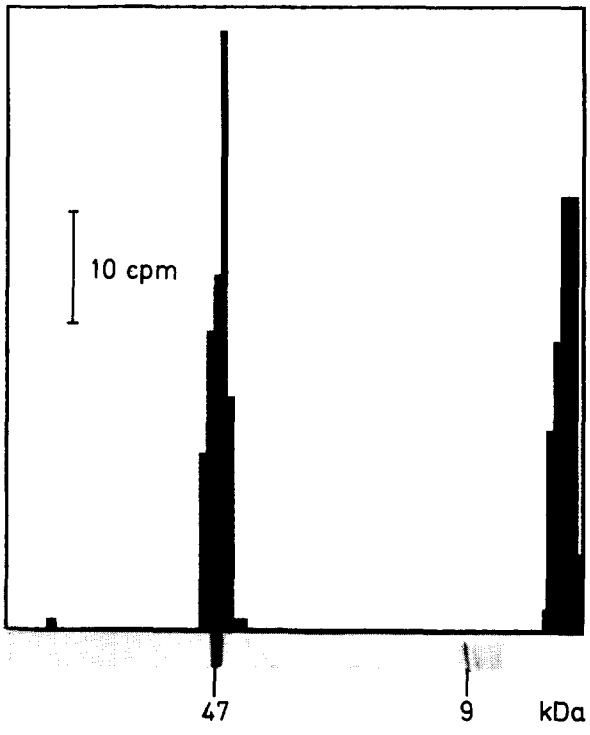

Fig. 7. Photograph of SDS-polyacrylamide gel electrophoresis gel (11.5–15%) and radioactivity distribution therein of a CP2-b preparation labeled with [^{3}H]azido-plastoquinone (7.7 nmol/mg Chl). [^{3}H]Azido-plastoquinone (0.514 mM, 0.2 μl) was added to 40 μl of CP2-b (366 μg Chl *a*/ml).

47 kDa polypeptide has a plastoquinone-binding site.

Discussion

Radioactive azido-atrazine has been used to identify the herbicide-binding protein of various plant materials [1–3]. In higher plants and algae, azido-atrazine [1–3], azido-triazinone [4] and azido-monuron [7] unambiguously label a 34 kDa polypeptide. The results obtained here demonstrate that the 28 kDa polypeptide of the *Synechococcus* PS II reaction center complex reacts not only with azido-atrazine, but also with azido-monuron and azido-dinoseb. The labeling of the 28 kDa polypeptide by all three azido-herbicides, which are considerably different in their molecular structure and the position of the azido group, provides a strong support for the conclusion that the 28 kDa polypeptide is the herbicide binding protein in *Synechococcus*. No labeling of the 28 kDa polypeptide by azido-atrazine was observed in the DCMU-insensitive CP2-b preparation, although the 28 kDa polypeptide is present in this preparation. The 28 kDa polypeptide or its surroundings must be modified in a way that the herbicide or the photoaffinity label cannot bind any longer. An identical observation was made in a *Chlamydomonas* PS II particle [12].

Previous experiments have shown that [^{3}H]azido-dinoseb labels predominantly a polypeptide(s) in the 40 to 53 kDa region in spinach and *Chlamydomonas* PS II preparations [11]. The use of *Synechococcus* PS II complexes enabled us to identify more specifically the polypeptide to which phenol-type herbicides bind. The observation that azido-dinoseb reacts with the 28 kDa polypeptide is consistent with the displacement experiments of radioactive 'DCMU-type' herbicides by phenol-type herbicides, which indicate that the phenol- and 'DCMU-type' herbicides share at least partly a common binding region [10]. Our results show that the 47 kDa polypeptide has a strong affinity for the azido-dinoseb. Because the 28 kDa polypeptide is associated with the 47 kDa polypeptide as evidenced by the isolation of CP2-b [15], the labeling of the 47 kDa polypeptide may reflect a situation that a dinoseb molecule bound to the 28 kDa polypeptide closely faces the 47 kDa polypeptide. It should be noted, however, that the 47 kDa polypeptide was heavily and specifically labeled in the CP2-b preparation, which mostly lacked the 28 kDa polypeptide (Fig .5). It is concluded, therefore, that the 47 kDa polypeptide has a binding site for the phenol-type herbicide.

Previous work with spinach thylakoids has suggested that [^{3}H]azide-plastoquinone tags a polypeptide in the 32–34 kDa region which migrates below the herbicide binding protein [12]. The identification of the quinone binding protein was considerably hampered by the ability of the azido-plastoquinone to react with various proteins, especially the light-harvesting chlorophyll *a/b* complexes, present in the htylakoids. It is important, therefore, to find conditions under which specific labeling of a small number of proteins can be realized. The photoaffinity labeling of

the *Synechococcus* PS II reaction center by azidoplastoquinone under the controlled conditions resulted in the incorporation of the radioactivity into the 47 kDa polypeptide and, to a lesser extent, into the 40 kDa polypeptide, whereas no radioactivity was found in the 28 kDa polypeptide which is considered to be the Q_B-binding protein and in other small polypeptides resolved from the complexes (Fig .6). In this case also ,the experiments with CP2-b provided evidence which strongly argue against the view that the plastoquinone analogue having the azido group at the end of a hydrophobic side chain binds to the 28 kDa polypetide, but labels the 47 kDa polypeptide rather than the 28 kDa polypeptide itself. The radioactivity was found exclusively in the 47 kDa polypeptide in the chlorophyll-protein complexes which have little or no 28 kDa polypeptide (Fig. 7). We conclude, therefore, that the 47 kDa polypeptide carries a plastoquinone-binding site. Q_A is present in the 47 kDa polypeptide (Satoh, K., unpublished data). The polypeptide may also carry Z which is assumed to be a bound plastoquinone. A possibility is that the quinone-binding site found here is a small fraction of the Q_A or Z site which is unoccupied by a plastoquinone molecule.

Acknowledgements

We thank Miss Fumiko Idei for her skillful technical assistance in the preparations of PS II samples. This work was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture of Japan, and from the Deutsche Forschungsgemeinschaft. Thanks are due to grants from Hitach Co. and Sonderforschungsbereich 168, which enabled K.S. to work at Ruhr-Universität, Bochum.

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