$[^{125}I]$Azido-Ioxynil Labels Val_{249} of the Photosystem II D-1 Reaction Center Protein

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Phenolic Herbicide, Photoaffinity Label, Amino Acid Sequencing, Photosystem II, D-1 Protein

Azido-ioxynil (3,5-diiodo-2-azido-4-hydroxy-benzonitrile) is a potent photosystem II inhibitor (pI_{50}-value 7.38) and as effective as the parent compound ioxynil itself. $[^{125}I]$azido-ioxynil exhibits specific binding to isolated thylakoids with a binding constant $K_b = 7.14$. Upon UV-illumination it binds covalently to thylakoids or photosystem II particles. It labels predominantly the 32 kDa D-1 photosystem II reaction center protein. A 41 kDa protein is only tagged in trace amounts. After proteolytic treatment of labeled D-1 protein with *Staphylococcus aureaus* V8-protease two major and two minor fragments are obtained. Automated gas phase sequencing of a 7 kDa cleavage peptide revealed that Val_{249} is the primary target of azido-ioxynil binding. Compared to urea type herbicides, this places the ioxynil binding site in a different environment of the D-1 photosystem II protein.

Introduction

Phenolic herbicides like dinitro-phenols or hydroxy-benzonitriles constitute a unique class among the photosystem II herbicides. Unlike other photosystem II inhibitors they do not have the common structural element $-N-C=X$ (where $X=O$ or N), they have a lag phase in binding [1], and they have a different π-charge distribution (for review, see [2]). Furthermore, weeds resistant against *s*-triazine herbicides are still susceptible towards phenolic herbicides, sometimes even more than the wild-type [3, 4]. An azido-derivative of the phenolic herbicide dinoseb, azido-dinoseb, preferentially labeled a protein of 41 kDa [5, 6]. Contrary, azido-derivatives of other photosystem II herbicides like *s*-triazine, triazinone or urea exclusively tag the 32 kDa D-1 photosystem II reaction center protein [7–9].

So far no other photolabile derivative of a phenolic herbicide was available to verify the labeling of the 41 kDa protein by phenolic herbicides. We wish to report here on the synthesis and properties of a $[^{125}I]$labeled 2-azido-derivative of the phenolic herbicide ioxynil (3,5-diiodo-4-hydroxy-benzonitrile). This compound marks the 41 kDa protein only in trace amounts, but preferentially binds to the 32 kDa D-1 photosystem II protein. Furthermore, by sequencing of a proteolytic fragment of the D-1 protein, labeled by $[^{125}I]$azido-ioxynil, Val_{249} was found to participate in the ioxynil binding niche in the photosystem II reaction center core complex. Azidomonuron was very recently found to bind to Tyr residues 237 and 254 in the D-1 protein [10]. Therefore, urea and phenol type herbicides bind with different orientations within the Q_B-site of the D-1 protein.

Materials and Methods

Synthesis

Azido-ioxynil (3,5-diiodo-2-azido-4-hydroxy-benzonitrile). 2.0 g (5.2 mmol) 3,5-diiodo-2-amino-4-hydroxy-benzonitrile [11] are finely pulverized and

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Abbreviations: chl, chlorophyll; CBB, Coomassie brilliant blue; DCIP, dichlorophenolindophenol; DNP-INT, 2-iodo-2′,4,4′-trinitro-3-methyl-6-isopropyldiphenylether; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulphate.

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suspended in 50 ml acetic acid. The compound dissolves at room temperature upon addition of 30 ml conc. H_2SO_4. It is diazotized at −5 °C by addition of an ice-cold solution of 0.8 g $NaNO_2$. Subsequently, a saturated solution of 0.8 g NaN_3 in ice-water is added and the reaction mixture stirred at 0 °C for 1 h. After dilution with ice-water, the precipitate is collected and recrystallized from ethanol. Yield: 1.29 g (60%). M.p.: dec. at 143 °C.

$C_7H_2I_2N_4O$ (412.2). Calc. C 20.39%, H 0.49%, N 13.59%, I 61.57%, O 3.88%.

Found: C 20.7%, H 0.3%, N 13,5%, J 61.2% O 4.1%.

[^{125}I]Azido-ioxynil (3,5-di[^{125}I]diiodo-2-azido-4-hydroxy-benzonitrile). 0.269 mg (2 μmol) 2-amino-4-hydroxybenzonitrile [11] in 100 μl 0.1 M phosphate buffer, pH 7.8, 0.9 mg (6 μmol) NaI in 100 μl phosphate buffer and 5 μl carrier-free $Na^{125}I$ (0.5 mCi; Amersham, Braunschweig, F.R.G.) are stirred in a brown, septum covered vial under an atmosphere of argon at room temperature. A solution of 1.82 mg (8 μmol) of chloramine-T in phosphate buffer is added in 10 μl portions at intervals of 5 min. The reaction mixture is then stirred for an additional hour. One drop of 6 N HCl is added and the reaction mixture several times extracted with ethyl acetate. The extract is concentrated *in vacuo* and chromatographed on silica gel precoated plastic sheets (Polygram SIL G/UV_{254}, Macherey-Nagel, Düren, F.R.G.) with benzene/2% methanol as the solvent. The zone, corresponding to the 3,5-diiodo-2-amino-4-hydroxybenzonitrile ($R_f = 0.60$) is cut out and eluted with methanol. The extract is transferred into a small vial and evaporated to dryness. 120 μl acetic acid and 80 μl conc. H_2SO_4 are added and the reaction mixture cooled to 0 °C. 0.138 mg (2 μmol) $NaNO_2$ in 10 μl H_2O are added in 2 μl portions at 10 min intervals, and then the reaction mixture is stirred for 1 additional hour. For conversion of the diazonium compound into the azide, 0.65 mg (10 μmol) NaN_3 in 10 μl H_2O are added in 2 μl portions at 5 min intervals. The reaction mixture is extracted several times with ethyl acetate, the ethyl acetate phase washed with water, dried over $MgSO_4$, concentrated *in vacuo* and chromatographed on silica gel precoated plastic sheets with ethyl acetate as the solvent. The zone, corresponding to the azide ($R_f = 0.77$) is cut out and eluted with methanol. The concentration of the azide was estimated from its A_{247} absorption maximum ($\varepsilon = 30.125$ M^{-1} cm^{-1}). The radiochemical yield was 448 nmol (23%) and the spec. activity 80 mCi/mmol.

Biochemical methods

Thylakoids from spinach were prepared according to [12] and photosystem II particles according to [13]. pI_{50}-values of azido-ioxynil in the system water > DCIP in the presence of DNP-INT [14] (to prevent DCIP photosystem I reduction) were estimated as described recently [15]. Binding experiments with [^{125}I]azido-ioxynil on thylakoids were carried out according to Tischer and Strotmann [16]. Photoaffinity labeling experiments were performed as in [5] except that a mercury lamp was used as light source. Separation of thylakoid proteins by polyacrylamide gel electrophoresis followed protocols either according to Chua [17] or Schägger *et al.* [18]. For autoradiography, dried gels were exposed for 3 days at −80 °C on Hyperfilm-MP (Amersham, Braunschweig, F.R.G.). For probing with antibodies against D-1 [19] and Nr. 268 D-2 [20] gels were transferred to nitrocellulose and immunoblotted [20]. For identification of labeled amino acids, D-1 protein was electroeluted from preparative gels, digested by *Staphylococcus aureus* V8-protease, the fragments again separated by preparative gel electrophoresis, electroeluted, and sequenced by automated gas-phase Edman degradation as in [10]. Aliquots of the phenylthiohydantoin amino acids were also assayed for radioactivity. The aliquots were sucked into small discs of filter paper, the discs dried and exposed to Hyperfilm-MP.

Results

Ioxynil is one of the most powerful photosystem II phenolic inhibitors and widely used as herbicide. pI_{50}-values of 7.3 [21] and 7.5 [1] have been reported. Introduction of an azido-group into the phenolic moiety does not change the inhibitory activity. The pI_{50}-value of azido-ioxynil was estimated in the system H_2O > DCIP in the presence of DNP-INT [14] to prevent photosystem I dependent DCIP-reduction [15]. With decreasing chl concentration, the pI_{50}-value of azido-ioxynil increases from 7.25 (20 μg chl) to 7.35 (5 μg chl) (Fig. 1). Extrapolated to 0 chl concentration, a pI_{50}-value of 7.38 is obtained (Fig. 1).

The binding curve of [^{125}I]azido-ioxynil to isolated thylakoids (Fig. 2, A) shows the same features of specific and unspecific binding as do other phenolic

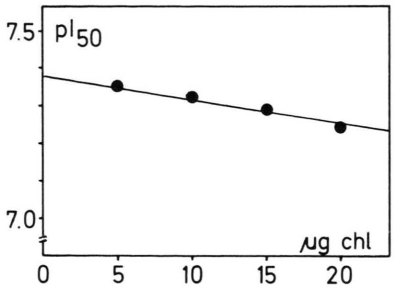

Fig. 1. Dependence from chl concentration of the pI$_{50}$-value of azido-ioxynil in photosynthetic DCIP-reduction.

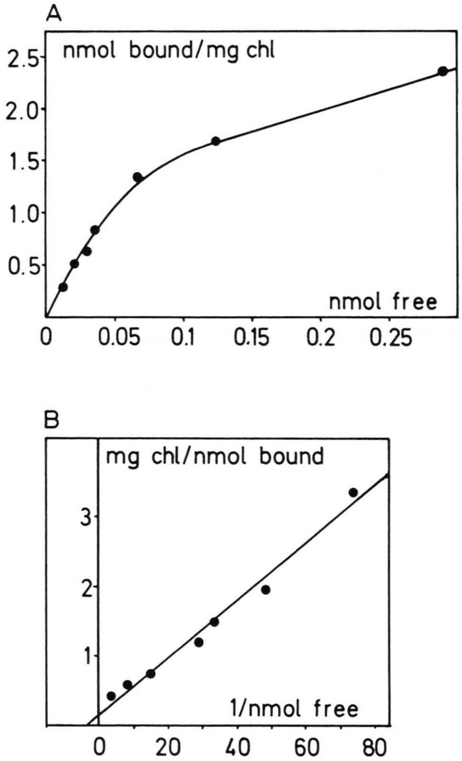

Fig. 2. Binding curve (A) and double-reciprocal plot (B) for binding of [^{125}I]azido-ioxynil to isolated thylakoids at pH 8.0.

herbicides [21]. From the Lineweaver-Burk plot of the binding data (Fig. 2, B) a binding constant $K_b = 117.9$ nM, corresponding to a $pK_b = 6.93$ can be obtained. This value is in good agreement with the pI$_{50}$-value of 7.38 (extrapolated to 0 chl concentration) and the middle-affinity binding constant of 7.14 for ioxynil [21], but somewhat less than the $pK_b = 8.0$ as obtained by prolonged preincubation for ioxynil [1]. The number of binding sites (x_t) was found to be 5.85 nmol/mg chl.

In a photoaffinity labeling experiment, [^{125}I]azido-ioxynil (5 nmol/mg chl) was covalently bound to the thylakoid membrane by UV-illumination. After separation on a 12% polyacrylamide gel in the presence of 6 M urea (Fig. 3, lane b) and autoradiography one single labeled protein of 32 kDa was detected (Fig. 3, lane c). It was identified as the photosystem II reaction center D-1 protein by probing with a D-1 antibody [19] (Fig. 3, lane d). The photosystem II reaction center D-2 polypeptide is not tagged, as judged by probing with the 268 D-2 antibody [20]. An identical result is obtained with photosystem II particles. Thus, azido-ioxynil labels the same protein as azido-derivatives of the *s*-triazine, triazinone or urea type [7–9]. It should be noted, however, that after prolonged autoradiography also a 41 kDa protein is found to be labeled, but to a much lesser extent than the D-1 protein (data not shown).

In order to get more information on the azido-ioxynil binding site and as a prerequisite for amino acid sequencing the labeled D-1 protein was subjected to Cleveland V8-protease digestion [22]. Fig. 4, lane d, shows the undigested D-1 protein,

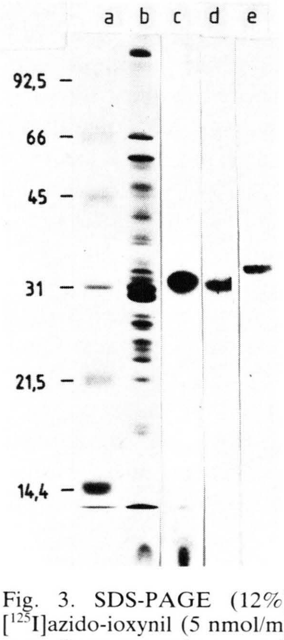

Fig. 3. SDS-PAGE (12%) of thylakoids labeled by [^{125}I]azido-ioxynil (5 nmol/mg chl). The gel contained 6 M urea. Lane a: marker proteins (kDa); lane b: CBB-staining; lane c: autoradiography; lane d: D-1 antibody; lane e: D-2 antibody.

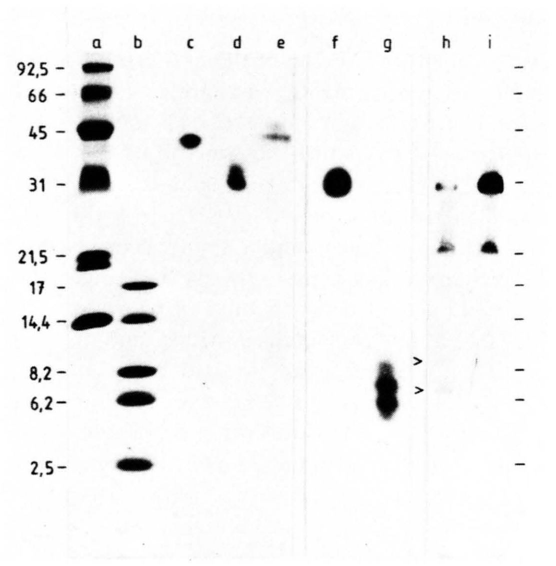

Fig. 4. SDS-PAGE (16%) of D-1 protein labeled by $[^{125}I]$azido-ioxynil and proteolytic cleavage products thereof (V8-protease). Lanes a, b: marker proteins (kDa); lane c, V8-protease; lane d: undigested D-1 protein; lane e: D-1 protein + V8-protease; lane f: autoradiography undigested D-1 protein; lane g: autoradiography D-1 protein + V8-protease; lane h: immunoblot D-1 protein + V8-protease, antibody against D-1 sequence Arg_{225}-Arg_{238}; lane i: immunoblot undigested D-1 protein, same antibody.

which is contaminated by a small amount of a 33 kDa protein, involved in the water splitting enzyme system ("Murata"-protein) [23]. In the CBB-stain of the digested D-1 protein (Fig. 4, lane e) no fragments of the D-1 protein are detectable, but only the V8-protease (compare lanes e and c). However, in the autoradiography (Fig. 4, lane g) four fragments can be seen: two major fragments with app. molecular masses of 7 kDa (fragment I) and 6 kDa (fragment II) and two minor fragments of 8 and 5 kDa. Lane f represents the autoradiography of the undigested D-1 protein. Lanes h and i, Fig. 4, show immunoblots of the digested (lane h) and undigested D-1 protein (lane i), respectively, of an antibody raised against a 14 amino acid long oligopeptide of the D-1 sequence [24] Arg_{225}-Arg_{238} and coupled to bovine serum albumin (W. Oettmeier, R. J. Berzborn and B. M. Greenberg, unpublished). In the undigested D-1 protein (lane i) a 23 kDa decomposition product of the D-1 protein is present, which may represent the natural D-1 degradation product [25]. This degradation product is also visible in the V8-protease digested sample (lane h) and, in addition, the 8 and 7 kDa (fragment I) proteins seem to react with the antibody (see arrows at lane h). The sequence Arg_{225}-Arg_{238} is located in a region immediately following transmembrane helix IV until a short parallel helix of the D-1 protein [26]. This suggests, that this sequence, which is known to be involved in herbicide binding [26] seems to be present in these two fragments.

When the isolation precedure for the D-1 protein was upscaled and the V8-protease treatment was carried out *in situ* in order to obtain pmole amounts of proteins for sequencing, the 7 kDa fragment I became the major degradation product. It was eluted from preparative gels and subjected to automated gas-phase Edman sequencing. The V8-protease cut took place at Glu_{244} (see also Fig. 6) and the sequence commenced at Thr_{245} (Fig. 5). Though the

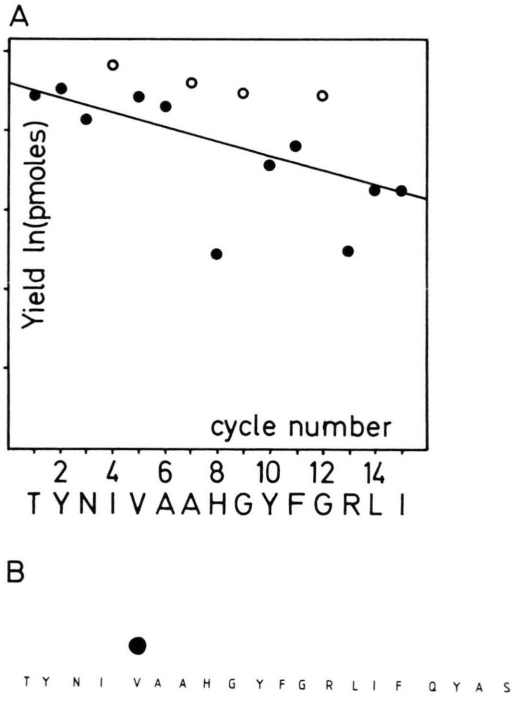

Fig. 5. (A) Sequence analysis of D-1 fragment I labeled by $[^{125}I]$azido-ioxynil. The ln of the yield of the phenylthiohydantoin derivatives in each cycle is plotted against the cycle number and the amino acid. Open dots symbolize yields which are extraordinarily high, because an identical amino acid was found in one of the polypeptides contaminating the D-1 fragment I. (B) Radioactivity assay of phenylthiohydantoin derivatives.

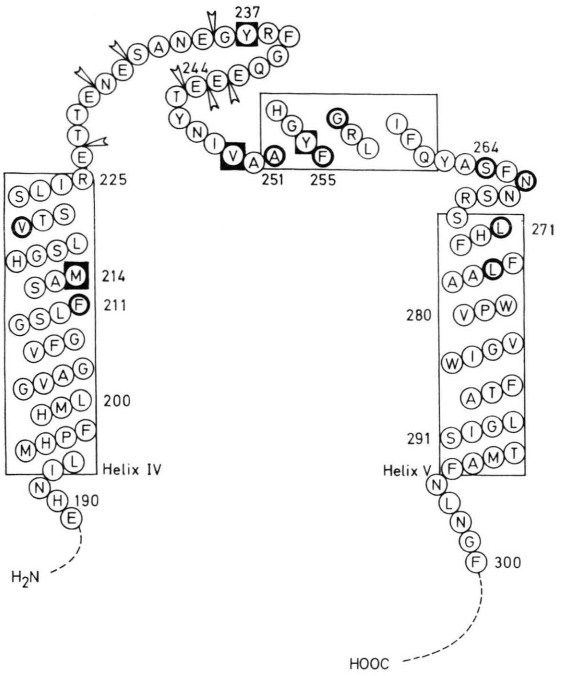

Fig. 6. Topography of the herbicide and Q_B-binding site of the D-1 protein of the photosystem II reaction center core complex. The two vertical boxes indicate the two transmembrane helices IV and V, the horizontal box indicates a helical part on top of the cytoplasmatic side of the membrane. Amino acids highlighted by thick circles represent places were mutations have been found to confer herbicide resistance. Amino acids where azido-labels bind are emphasized by rectangular boxes. Possible cutting sites by V8-protease are indicated by arrows.

7 kDa fragment was contaminated by three cleavage products of the V8-protease itself and part of the 33 kDa "Murata"-protein [23], the sequence of the D-1 protein could be clearly read until Ile_{259} (Fig. 5A). Sequencing was continued for 5 additional cycles until Ser_{264}. When aliquots of the phenylthiohydantoin amino acids were assayed for radioactivity, the main radioactivity was localized in Val_{249} and a trace in the adjacent Ala_{250} (Fig. 5B). This result identifies Val_{249} as the main target of the dehydroazepine generated from azido-ioxynil by UV-illumination. It should be noted that the polybrene support in the sequencing machine after the last cycle still contained some radioactivity, which might indicate that an additional amino acid downstream in the sequence is labeled.

Discussion

Fig. 6 depicts the region of the D-1 protein which is mainly responsible for Q_B and herbicide binding. It consists of transmembrane helices IV and V and a short parallel helix which lies on top of the membrane [26]. Certain amino acids whose substitution will lead to herbicide resistance [27] are highlighted by thick circles. Consequently, amino acids involved in herbicide binding cover at least the range from Phe_{211} to Leu_{275}, which is a total of 65 amino acid residues. Within this sequence, Met_{214} and an additional, yet unidentified amino acid between His_{215} and Arg_{225} are thought to be involved in azido-atrazine binding [28]. Most recently, Tyr_{237} and Tyr_{254} were shown to be the targets for azido-monuron by direct sequencing [10]. The results of this paper have identified Val_{249} as one amino acid involved in ioxynil binding. In particular, the 2-position of azido-ioxynil must point towards this amino acid. This will place the ioxynil binding site in a different environment of the D-1 protein as compared to urea type herbicides. Different binding sites have also been found for the inhibitors terbutryn (a *s*-triazine) and *o*-phenanthroline [29]. *o*-Phenanthroline binds closely to the nonheme iron and its nitrogen atoms are in close contact with His_{190} in the L-subunit (which is equivalent to His_{215} in D-1). The binding site of terbutryn is further away from the non-heme iron. It is bonded to Ile_{224} and Ser_{223} (corresponding to Asn_{266} and Ser_{264} in D-1). Based on this result, a similar proposal was made for the binding of phenols and urea type herbicides in photosystem II [26]. Urea type inhibitors were classified as the Ser_{264}-family, whereas phenols were classified as the His_{215}-family, according to their different biochemical properties. Cyanophenols like ioxynil should bind like the other phenols, but not necessarily strongly to His_{215} [26].

In a most recent work, in a ioxynil-insensitive mutant of the blue-green alga *Synechocystis* 6714, Asn_{266} was found to be exchanged against Thr [30]. If one considers the position of Val_{249} and Asn_{266} in a three-dimensional model of the D-1 protein, built according to its homology with the L-subunit of the bacterial photosynthetic reaction center [31], both amino acids are found to be located in close vicinity to each other. It is easily imaginable that ioxynil in its binding niche is sandwiched between Val_{249} and Asn_{266}. This will indeed place the ioxynil binding region in an environment in the D-1 protein which is different from that of urea type inhibitors, and also remote from His_{215}. This is the major reason for the biochemical differences found between both types of herbicides [1–5].

Acknowledgements

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[1] A. Thiel and P. Böger, Pestic. Biochem. Physiol. **25,** 270 (1986).
[2] W. Oettmeier and A. Trebst, in: "The Oxygen Evolving System of Photosynthesis" (Y. Inoue, N. Murata, A. R. Crofts, G. Renger, Govindjee, and K. Satoh, eds.), p. 411, Academic Press, Inc., Tokyo 1983.
[3] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34c,** 996 (1979).
[4] W. Oettmeier, K. Masson, C. Fedtke, J. Konze, and R. R. Schmidt, Pestic. Biochem. Physiol. **18,** 357 (1982).
[5] W. Oettmeier, K. Masson, and U. Johanningmeier, FEBS Lett. **118,** 267 (1980).
[6] W. Oettmeier, K. Masson, and U. Johanningmeier, Biochim. Biophys. Acta **679,** 376 (1982).
[7] K. Pfister, K. E. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **78,** 981 (1981).
[8] W. Oettmeier, K. Masson, H. J. Soll, and W. Draber, Biochim. Biophys. Acta **767,** 590 (1984).
[9] A. Boschetti, M. Tellenbach, and A. Gerber, Biochim. Biophys. Acta **810,** 12 (1985).
[10] R. Dostatni, H. E. Meyer, and W. Oettmeier, FEBS Lett. **239,** 207 (1988).
[11] H. W. Schmidt, Synthesis **1985,** 778.
[12] N. Nelson, Z. Drechsler, and J. Neumann, J. Biol. Chem. **245,** 143 (1970).
[13] D. A. Berthold, G. T. Babcock, and C. F. Yocum, FEBS Lett. **134,** 231 (1981).
[14] A. Trebst, H. Wietoska, W. Draber, and H. J. Knops, Z. Naturforsch. **33c,** 919 (1978).
[15] W. Oettmeier, D. Godde, B. Kunze, and G. Höfle, Biochim. Biophys. Acta **807,** 216 (1985).
[16] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113 (1977).
[17] N. H. Chua, Meth. in Enzymology **69,** 434 (1980).
[18] H. Schägger, U. Borchart, H. Aquila, T. A. Link, and G. v. Jagow, FEBS Lett. **190,** 89 (1985).
[19] U. Johanningmeier, Z. Naturforsch. **42c,** 755 (1987).
[20] R. Geiger, R. J. Berzborn, B. Depka, W. Oettmeier, and A. Trebst, Z. Naturforsch. **42c,** 491 (1987).
[21] W. Oettmeier, C. Kude, and H. J. Soll, Pestic. Biochem. Physiol. **27,** 50 (1987).
[22] D. W. Cleveland, S. G. Fischer, M. W. Kirschner, and U. K. Laemmli, J. Biol. Chem. **252,** 1102 (1977).
[23] H. Oh-oka, S. Tanaka, K. Wada, T. Kuwabara, and N. Murata, FEBS Lett. **197,** 63 (1986).
[24] G. Zurawski, H. J. Bohnert, R. R. Whitfeld, and W. Bottomley, Proc. Natl. Acad. Sci. U.S.A. **79,** 7699 (1982).
[25] B. M. Greenberg, V. Gaba, A. K. Mattoo, and M. Edelman, EMBO J. **6,** 2865 (1987).
[26] A. Trebst, Z. Naturforsch. **42c,** 742 (1987).
[27] J. D. Rochaix and J. Erickson, TIBS **13,** 56 (1988).
[28] P. K. Wolber, M. Eilmann, and K. E. Steinback, Arch. Biochem. Biophys. **248,** 224 (1986).
[29] H. Michel, O. Epp, and J. Deisenhofer, EMBO J. **5,** 2445 (1986).
[30] G. Ajlani, I. Meyer, C. Vernotte, and C. Astier, FEBS Lett., in press.
[31] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, Nature **318,** 618 (1985).