Accelerated Publications

Ciprofloxacin and Etoposide (VP16) Produce a Similar Pattern of DNA Cleavage in a Plasmid of an Archaebacterium†

Mouldy Sioud‡ and Patrick Forterre*
Institut de Microbiologie, Université Paris-Sud, Centre d'Orsay, 91405 Orsay Cedex, France
Received January 4, 1989; Revised Manuscript Received February 7, 1989

ABSTRACT: The fluoroquinolone ciprofloxacin, an inhibitor of eubacterial DNA gyrase, induces single- and double-stranded DNA breaks in the plasmid pGRB-1 from the halophilic archaebacterium *Halobacterium* GRB when the cells are treated by this drug in a magnesium-depleted medium. This reaction is prevented by a dose of novobiocin known to specifically inhibit DNA gyrase. Cleavage of pGRB-1 DNA induced by either ciprofloxacin or the antitumoral drug etoposide (VP16) produces DNA fragments of identical lengths. These results indicate that ciprofloxacin, novobiocin, and etoposide have a common target in *Halobacterium* GRB: an archaebacterial type II DNA topoisomerase. The similarity of DNA cleavage patterns induced by ciprofloxacin and etoposide is a new and strong argument that quinolone and epipodophyllotoxins have the same mode of interaction with the DNA–DNA topoisomerase II complexes. The plasmid pGRB-1 could be used to prescreen in the same system both antibiotics that inhibit bacterial gyrase and antitumoral drugs that inhibit eukaryotic DNA topoisomerase II.

DNA topoisomerases II catalyze the crossing of two DNA double helices via a transient double-stranded break in one of the helices [for reviews on DNA topoisomerases, see Maxwell and Gellert (1986), Vosberg (1985), and Wang (1985, 1987)]. Together with DNA topoisomerases I, DNA topoisomerases II determine the supercoiling density of the DNA and solve the topological problems generated by the opening of the double helix during DNA replication, transcription, and recombination. In addition, DNA topoisomerases II are specifically required to unlink daughter DNA molecules during chromosome segregation, and the eukaryotic DNA topoisomerase II is a structural component of the nuclear chromosomes.

The biological importance of DNA topoisomerases II is emphasized by the existence of numerous antibiotics inhibiting these enzymes (Drlica & Franco, 1988). The eukaryotic enzyme is the target of several families of natural or semisynthetic antitumoral drugs: anthracyclines, acridines, ellipticines, and epipodophyllotoxins (Tewey et al., 1984; Ross et al., 1984). All these compounds are also DNA intercalators with the exception of the epipodophyllotoxins. The eubacterial DNA topoisomerase II (DNA gyrase) is specifically inhibited by low doses of coumarin antibiotics (1–10 μg/mL) (Gellert et al., 1976) whereas at higher doses (>100 μg/mL) these drugs also inhibit the eukaryotic DNA topoisomerase II (Hsieh & Brutlag, 1980). In addition, DNA gyrase is the target of quinolones, a family of synthetic antibiotics widely used in antibiotherapy (Gellert et al., 1977; Sugino et al., 1977). The quinolones have no effect on eukaryotic type II DNA topoisomerase (Hsieh & Brutlag, 1980). Nevertheless, the mechanisms of action of quinolones on DNA gyrase and of antitumoral drugs on eukaryotic DNA topoisomerase II have striking similarities. All these drugs prevent the ligation step of the topoisomerization reaction; consequently, they induce the accumulation of reaction intermediates in which the DNA topoisomerase II is covalently linked to the 5′ ends of the DNA breaks and bound to the 3′ ends via noncovalent interactions. These reaction intermediates can be revealed if the cells are lysed in the presence of a protein denaturant, since the cleaved DNA strands are separated by the unfolding of the topoisomerase. Both single- and double-stranded breaks are observed, probably because the cleavage–ligation mechanism does not occur at the same time on both DNA strands, thus producing "cleavable complexes" in which either one or two DNA strands are interrupted (Snyder & Drlica, 1979).

The common feature of the action of quinolones and antitumoral drugs has suggested that both compounds have a similar mode of interaction with the type II DNA topoisomerase–DNA complexes (Nelson et al., 1984). Comparison between quinolones and antitumoral drugs of the epipodophyllotoxin family (etoposide and teniposide) has been considered especially relevant, since neither type of drug is a DNA intercalator (Chen et al., 1984). Further comparison between the mode of action of quinolones and epipodophyllotoxins has been hampered by the lack of a living system sensitive to both types of drugs. We have considered the possibility of bypassing this difficulty by using an archaebacterial system. Indeed, we have previously shown that archaebacteria are sensitive to both inhibitors of eukaryotic and eubacterial DNA topoisomerases II (Forterre et al., 1986), an observation that correlates well with the intermediate phylogenetic position of archaebacteria between eukaryotes and "classical bacteria" or eubacteria (Woese, 1987).

Among the archaebacteria, the halobacteria are the most sensitive organisms to DNA topoisomerase II inhibitors (Forterre et al., 1986; Sioud et al., 1988b): They are sensitive both to antitumoral drugs active on the eukaryotic enzyme and to low doses of coumarins which are otherwise specific for inhibition of the eubacterial DNA gyrase. The neutrophilic halobacteria are resistant to quinolones (Forterre et al., 1986), including the fluoroquinolones, a family of very potent qui-

† This work was supported by a grant from the Association de la Recherche sur le Cancer.
‡ Present address: Institut of Immunology and Rheumatology, Rikshospitalet Fr. Quamsgt. 1, 0172 Oslo 1, Norway.

0006-2960/89/0428-3638$01.50/0

nolone derivatives that strongly inhibit DNA gyrase but not eukaryotic DNA topoisomerase II (Hussy et al., 1986). In contrast, the alkaliphilic halobacteria *Natronobacterium gregoryi* is sensitive to fluoroquinolones (Forterre et al., 1986).

In a previous work, we have used the small plamid pGRB-1 of the strain *Halobacterium* GRB as a probe to study the mode of action of coumarins and epipodophyllotoxins on DNA topology in archaebacteria (Sioud et al., 1987b, 1988a). We have found that these drugs have similar effects in archaebacteria as in the other two primary kingdoms; i.e., the coumarin novobiocin induces positive supercoiling as in eubacteria whereas the epipodophyllotoxin etoposide (VP16) induces single- and double-stranded DNA breaks with a protein covalently linked to their 5′ ends as in eukaryote. Unfortunately, it was not possible a priori to study the effect of fluoroquinolones on pGRB-1 because *Halobacterium* GRB is a neutrophilic halobacteria and is therefore resistant to fluoroquinolones. Furthermore, the two strains of archaebacteria known to be sensitive to these drugs (*N. gregoryi* and *Methanosarcina barkerii*; Sioud et al., 1988b) lacked any small plasmids that are easy to manipulate for topological analyses.

We have previously suggested that the resistance of neutrophilic halobacteria to fluoroquinolones could be due to the high magnesium concentration (20 g/L) of their culture medium (Forterre et al., 1986). This hypothesis was supported by two observations: (1) alkaliphilic halobacteria are phylogenetically closely related to neutrophilic ones (Tindall et al., 1984), the main phenotypic difference between them (besides their pH dependence) being that the former require a much lower magnesium concentration for growth (<1 g/L); (2) we observed that a halotolerant eubacteria, *Halomonas elongata*, was highly sensitive to quinolones when grown in LB medium but completely resistant to these drugs when this medium was supplemented with 20 g/L magnesium (P. Forterre, unpublished observation). These considerations caused us to find out whether it was possible to test the effect of fluoroquinolones on pGRB-1 topology by simply removing the magnesium from the culture medium at the time of drug addition.

Materials and Methods

The cells of *Halobacterium* GRB (kindly provided by U. Rdest, Wurzburg, RFA) were cultivated at 37 °C in the classical halophile medium (CHM), which contains 20 g/L $MgCl_2$ [see composition of the medium in Sioud et al. (1987b)]. At OD = 0.4, the cells were harvested and resuspended for 16 h either in complete CHM medium or in CHM medium without magnesium, in the presence or absence of drugs. Topological analysis of the pGRB-1 plasmid was performed according to Sioud et al. (1987b); briefly, the cells were lysed in the presence of the protein denaturant sodium dodecyl sulfate (SDS) and proteinase K, and the cleared lysates were electrophoresed on one- or two-dimensional agarose gels. The DNA were transferred onto nitrocellulose and hybridized with a ^{32}P-labeled pGRB-1 probe.

Results and Discussion

The transfer of *Halobacterium* GRB exponentially growing cells in classical halophile medium (CHM) depleted of magnesium induced the arrest of growth and changed the cell morphology from irregular rod shaped to regular round shaped. Nevertheless, we did not detect loss of cell viability, nor topological change of the pGRB-1 plasmid, at least up to 48 h after the initiation of magnesium depletion. The growth resumed after addition of magnesium, indicating that the effects induced by magnesium depletion are reversible.

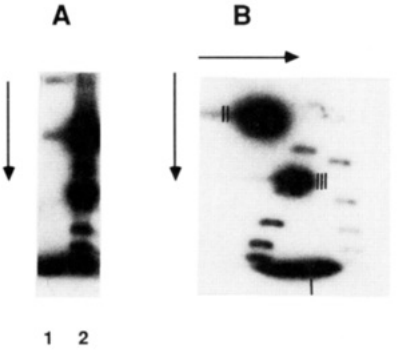

FIGURE 1: Ciprofloxacin induces single- and double-stranded breaks in the plasmid pGRB-1 in the absence of magnesium. Panel A: One-dimensional agarose gel electrophoresis of pGRB-1 isolated from cells treated with 50 μg/mL ciprofloxacin in the presence (lane 1) and in the absence (lane 2) of magnesium. Panel B: Two-dimensional agarose gel electrophoresis of the same sample as in lane 2; (I) negatively supercoiled DNA form I, (II) nicked DNA, and (III) linear DNA.

We then looked at the effects of the toposiomerase II inhibitors VP16 and novobiocin on plasmid topology in the absence of magnesium. We observed that these effects were similar to those previously described in the presence of magnesium (Sioud et al., 1987b, 1988a); VP16 produces the appearance of nicked and linear plasmid DNA and a ladder of both negatively and positively supercoiled topoisomers whereas novobiocin produces a ladder of positively supercoiled DNA molecules (not shown). These results indicated that magnesium depletion of the culture medium at the time of drug addition did not inhibit the intracellular action of the halobacterial type II DNA topoisomerase nor its response to Vp16 and novobiocin. We then tested, under the same conditions, the effect of the fluoroquinolone ciprofloxacin on pGRB-1 topology. Figure 1, panel A, shows that ciprofloxacin has no effect on pGRB-1 topology when added in the complete medium (lane 1) but produces a striking effect when added to the culture medium after magnesium depletion (lane 2). The various new DNA bands appearing after ciprofloxacin treatment were identified by analysis on two-dimensional gel electrophoresis. Figure 1, panel B, shows that these new bands correspond mainly to nicked (form II) and linear plasmid molecules (form III) and a lower amount of relaxed topoisomers with both negatively and positively supercoiled molecules (left and right branches of the arch, respectively). The minimal concentrations of ciprofloxacin which induced DNA cleavage were in the range 2–5 μg/mL (Figure 2), similar to the minimal doses affecting in vivo the growth of the alkaliphilic halophile *N. gregoryi* (Sioud et al., 1988a). The amount of DNA cleavage increased with ciprofloxacin concentrations up to 25 μg/mL (Figure 2). At higher doses, the ratio of cleaved to intact plasmids remained constant (not shown). These results were similar to those obtained with VP16 (Sioud et al., 1987), except that the amount of cleaved DNA was somewhat greater in the case of ciprofloxacin.

We have previously shown that low doses of novobiocin (1–2 μg/mL) partly antagonize the effect of ciprofloxacin on the growth of *N. gregoryii* (Sioud et al., 1988a). Figure 2 (lane 6) shows that ciprofloxacin-induced DNA cleavage in *Halobacterium* GRB was also inhibited by novobiocin, and at a similar concentration (2 μg/mL). Recently, it has been shown that coumermycin, an analogue of novobiocin, also blocks the cleavage activity of oxolinic acid, an analogue of ciprofloxacin, in eubacteria (Franco & Drlica, 1988). These results are reminiscent of the effect of novobiocin on VP16-induced DNA cleavage in eukaryotes and archaebacteria. In eukaryotes,

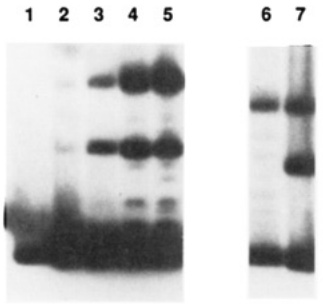

FIGURE 2: Effect of ciprofloxacin concentrations and novobiocin treatment on ciprofloxacin-induced pGRB-1 DNA cleavage. Lane 1: pGRB-1 isolated from *Halobacterium* GRB cells after 16 h of suspension in CHM medium without magnesium in the absence of drug. Lanes 2–5: pGRB-1 isolated from cells treated with 2, 5, 10, and 25 μg/mL ciprofloxacin, respectively. Lane 6: pGRB-1 isolated from cells treated with 5 μg/mL ciprofloxacin and 2 μg/mL novobiocin. Lane 7: Same as lane 6 without novobiocin treatment.

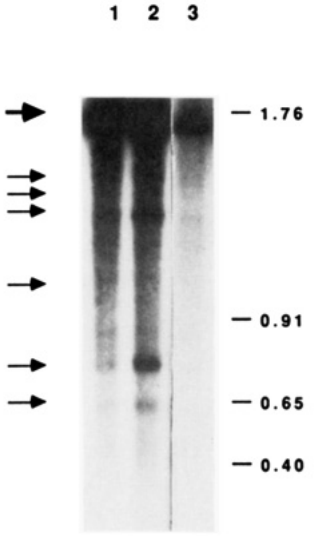

FIGURE 3: Comparison of DNA cleavage patterns induced by VP16 and ciprofloxacin in the pGRB-1 plasmid. *Halobacterium* GRB cells were treated in CHM medium without magnesium for 16 h with 50 μg/mL VP16 or ciprofloxacin. Total DNA was extracted and treated by the restriction enzyme *Sal*I. The restricted DNA was run on agarose gel and hybridized with a ^{32}P-labeled pGRB-1 probe. Preparation of the DNA and electrophoresis were as described by Sioud et al. (1987b). Lane 1, restricted DNA from VP16-treated cells; lane 2, restricted DNA from ciprofloxacin-treated cells; lane 3, restricted DNA from untreated cells. Broad arrow, position of linearized plasmids; thin arrows, major cleavage sites induced by ciprofloxacin and VP16.

novobiocin inhibits in vivo and in vitro DNA cleavage induced by the antitumoral drugs active on the DNA topoisomerase II (Marshall et al., 1983; Yang et al., 1985), whereas we have previously demonstrated that novobiocin inhibits VP16-induced chromosomal DNA cleavage in *Halobacterium halobium* (Sioud et al., 1987a) and VP16-induced pGRB-1 cleavage in *Halobacterium* GRB (Sioud et al., 1987b).

The modification of plasmid topology induced by ciprofloxacin in *Halobacterium* GRB in magnesium-depleted medium is a new and strong argument in favor of our previous hypothesis, i.e., that the resistance of neutrophilic halobacteria to fluoroquinolones is due to the high magnesium concentration of the culture medium (Forterre et al., 1986). Furthermore, the similarity between DNA cleavage induced by the quinolone oxolinic acid in eubacteria via its action on DNA gyrase and DNA cleavage induced by ciprofloxacin in halobacteria is the first evidence that the target of fluoroquinolones in archaebacteria is a type II DNA topoisomerase. This conclusion is strengthened by the sensitivity of ciprofloxacin-induced DNA cleavage to the coumarin novobiocin at a dose which is otherwise specific for the inhibition of bacterial DNA gyrase.

The production of double-stranded breaks by VP16 and ciprofloxacin in the same DNA molecule in vivo allows us to compare the sites of action of these two drugs at the DNA level. The total DNA extracted from *Halobacterium* GRB cells treated by either VP16 or ciprofloxacin was digested by the restriction enzyme *Sal*I, which has a single cutting site on the pGRB-1 plasmid. The digested plasmid fragments were specifically detected by the pGRB-1 probe used in our experiments, since this probe does not hybridize to chromosomal DNA of *Halobacterium* GRB (Sioud et al., 1987b). Forms I and II, as well as the various DNA topoisomers obtained after the drug action, produced linear plasmid molecules upon treatment with *Sal*I. In contrast, the plasmids previously linearized by the drugs produced DNA fragments of different sizes according to the location of the DNA topoisomerase II–DNA complex relative to the *Sal*I restriction site. The pattern of DNA fragmentation after VP16 treatment was similar in the absence and in the presence of magnesium (not shown). Figure 3 shows that, in the absence of magnesium, VP16 and ciprofloxacin treatment produced the same sets of DNA fragments with identical sizes. The relative abundance of the different fragments was also similar except for a 0.75-kb fragment, which is more abundant in the ciprofloxacin-treated sample. This finding indicates that VP16 and ciprofloxacin trap the reaction intermediates between the archaebacterial DNA topoisomerase II and pGRB-1 DNA at the same sites on this plasmid and that most of the different cleavable complexes are formed with the same efficiency by the two types of drug. The relative amount of each DNA fragment recovered after drug treatment may reflect either the amount of DNA topoisomerase II fixed on different in vivo binding sites or different sensitivities of DNA topoisomerase II molecules to the drugs according to their location on the plasmid.

In eukaryotes, the relative efficiency of cleavage observed at different DNA topoisomerase II binding sites in vitro varies with different families of antitumoral drugs (Tewey et al., 1984). It is not clear why different anticarcinogenic agents produce different cleavage patterns (Drlica & Franco, 1988). Interestingly, in eubacteria, the sites on DNA where gyrase induces DNA cleavage are identical whether or not the quinolone oxolinic acid is present (Fisher et al., 1981, 1986). This also seems to be the case in archaebacteria since two fragments can be detected in tiny amounts after digestion of the DNA from control cells that correspond to the two major fragments obtained with VP16 or ciprofloxacin (Figure 3, lane 3). They could correspond to physiological cleavable complexes trapped at the time of cell lysis.

An interesting hypothesis is that DNA topoisomerase II inhibitors, which are also DNA intercalators, change the relative binding affinity of this enzyme for different sites, whereas drugs that do not intercalate into the DNA, such as quinolones and epipodophyllotoxins, trap DNA topoisomerases II specifically at their physiological sites of action. In the case of quinolones, this would be in agreement with the model of action proposed by Shen, in which these drugs bind to a single-stranded pocket of DNA formed during the strand passage reaction catalyzed by gyrase (Drlica & Franco, 1988); our finding suggests that this model is also valuable for the mode of interaction of epipodophyllotoxins with topoisomerase II–DNA complexes.

This paper should encourage the use of previous data obtained on the action of quinolones in bacteria for further

investigation in eukaryotes. It may be also relevant for drug testing. We have previously suggested that the archaebacterial plasmid pGRB-1 could be useful in the prescreening of antitumoral drugs active on DNA topoisomerases II, because topological changes induced by VP16 in this plasmid are easy to monitor (Sioud et al., 1987b). Our new finding indicates that this system could be used at the same time to look for new antibiotics active against bacterial DNA gyrase. The main advantage of such in vivo prescreening would be the identification of topoisomerase inhibitors in complex mixtures of natural products containing nuclease activities preventing the testing of topoisomerase activities in vitro.

ACKNOWLEDGMENTS

We thank A. M. De Recondo and G. Baldacci for support and discussions and C. Jaxel for the gift of ciprofloxacin.

Registry No. Ciprofloxacin, 85721-33-1; etoposide, 33419-42-0; DNA topoisomerase, 80449-01-0; quinolone, 13721-01-2; epipodophyllotoxins, 4375-07-9.

REFERENCES

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560-13566.
Drlica, K., & Franco, R. J. (1988) *Biochemistry 27*, 2253-2259.
Fisher, L. M., Mizuuchi, K., O'Dea, M. H., Ohmori, H., & Gellert, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4165-4169.
Fisher, L. M., Barot, H. A., & Cullen, M. E. (1986) *EMBO J. 5*, 1411-1418.
Forterre, P., Nadal, M., Elie, C., Mirambeau, G., Jaxel, C., & Duguet, M. (1986) *Syst. Appl. Microbiol. 9*, 77-82.
Franco, R. J., & Drlica, K. (1988) *J. Mol. Biol. 201*, 229-233.
Gellert, M., O'Dea, M. H., Itoh, T., & Tomizawa, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4474-4478.
Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., & Tomizawa, j. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4772-4776.
Hsieh, T., & Brutlag, D. (1980) *Cell (Cambridge, Mass.) 21*, 115-125.
Hussy, P., Maass, G., Tummler, B., Grosse, F., & Schomburg, U. (1986) *Antimicrob. Agents Chemother. 29*, 1073-1078.
Marshall, B., Darkin, S. J., & Ralph, R. (1983) *FEBS Lett. 161*, 75-78.
Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem. 38*, 69-107.
Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361-1365.
Ross, W., Rowe, T., Glisson, B., Yalowitz, J., & Liu, L. F. (1984) *Cancer Res. 44*, 5857-5860.
Sioud, M., Baldacci, G., Forterre, P., & De Recondo, A. M. (1987a) *Eur. J. Biochem. 169*, 231-236.
Sioud, M., Baldacci, G., Forterre, P., & De Recondo, A. M. (1987b) *Nucleic Acids Res. 15*, 8217-8234.
Sioud, M., Possot, O., Elie, C., Sibold, L., & Forterre, P. (1988a) *J. Bacteriol. 170*, 946-953.
Sioud, M., Baldacci, G., De Recondo, A. M., & Forterre, P. (1988b) *Nucleic Acids Res. 16*, 1379-1391.
Snyder, M., & Drlica, K. (1979) *J. Mol. Biol. 131*, 287-302.
Sugino, A., Peebles, C., Kreuzer, K., & Cozzarelli, N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4838-4842.
Tewey, K., Rowe, R., Yand, L., Nelson, E. M., & Liu, L. F. (1984) *Science (Washington, D.C.) 226*, 446-468.
Tindall, B. J., Ross, H. N. M., & Grant, W. D. (1984) *Syst. Appl. Microbiol. 5*, 41-57.
Vosberg, H. P. (1985) *Curr. Top. Microbiol. Immunol. 114*, 19-100.
Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665-697.
Wang, J. C. (1987) *Biochim. Biophys. Acta 909*, 1-9.
Woese, C. R. (1987) *Microbiol. Rev. 51*, 221-271.
Yang, L., Rowe, T. C., Nelson, E. M., & Liu, L. F. (1985) *Cancer Res. 45*, 5872-5876.

A Resonance Raman Characterization of the Primary Electron Acceptor in Photosystem II

Pierre Moënne-Loccoz, Bruno Robert,* and Marc Lutz
Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif sur Yvette Cedex, France
Received January 25, 1989; Revised Manuscript Received February 28, 1989

ABSTRACT: Low-temperature resonance Raman spectra of D1/D2 particles from spinach excited at 406 and 413 nm contained enhanced contributions from pheophytin *a*. These contributions were partly photobleachable in dithionite-treated particles. Difference resonance Raman spectra calculated on this basis essentially arose from a single environmental population of neutral pheophytin *a*. The 9-keto carbonyls of these bleachable molecules vibrated at 1680 cm^{-1}, a frequency identical, within experimental uncertainty, with that of the 9-keto carbonyl of the acceptor bacteriopheophytin H_L in the reaction centers of *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*. This constitutes strong evidence that the acceptor pheophytin of the PS II reaction center is H-bonded to the D1-130 glutamic residue, in a relative geometry and environment that must be very close to those of the H_L molecule and the L-104 glutamic residue in the reaction centers of the above two bacterial species.

In photosystem II (PS II) of oxygenic photosynthetic organisms, the sites of the primary electron-transfer steps have been shown to be located in two polypeptides of mass 39 and 39.5 kDa, named D1 and D2, respectively. Indeed, Nanba and Satoh (1986) recently isolated a photosystem II complex from spinach that only contained the D1 and D2 polypeptides

0006-2960/89/0428-3641$01.50/0