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Evaluation of Selected Benzoquinones, Naphthoquinones, and Anthraquinones as Replacements for Phylloquinone in the A_1 Acceptor Site of the Photosystem I Reaction Center†

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Received March 12, 1990; Revised Manuscript Received April 25, 1990

ABSTRACT: Selected substituted 1,4-benzoquinones, 1,4-naphthoquinones, and 9,10-anthraquinones were investigated as possible replacement quinones in spinach photosystem I (PSI) preparations that had been depleted of endogenous phylloquinone by extraction with hexane/methanol. As a criterion for successful biochemical reconstitution, the restoration of electron transfer was determined by measuring P-430 turnover at room temperature from flash-induced absorbance transients. Restoration of complete electron transfer between A_0^- and P-430 (terminal iron–sulfur centers, F_AF_B) was demonstrated by using phylloquinone, 2-methyl-3-decyl-1,4-naphthoquinone, 2-methyl-3-(isoprenyl)$_2$-1,4-naphthoquinone, and 2-methyl-3-(isoprenyl)$_4$-1,4-naphthoquinone. All other quinones tested did not restore P-430 turnover but acted as electron acceptors and oxidized A_0^-. It is concluded that the specificity of the replacement quinone for interaction with the primary acceptor, A_0^-, is low but additional structural constraints are required for the quinone occupying the A_1 site to donate to the iron–sulfur center, F_x. It is suggested that the 3-phytyl side chain of phylloquinone and the 3-alkyl tails of the three naphthoquinones that restored P-430 turnover may be required for interaction with a hydrophobic domain of the A_1 site in the PSI core to promote electron transfer to F_x and then to F_AF_B.

In green plant and cyanobacterial photosynthesis, the primary charge separation in photosystem 1 (PSI)[1] ($P\text{-}700^+A_0^-$) is stabilized by successive electron transfers through the redox centers referred to as A_1, F_x, and F_AF_B (Golbeck, 1987). This report is concerned with the center A_1, which was originally postulated to be a quinone on the basis of EPR measurements (Gast et al., 1983; Thurnauer & Gast, 1985). Their provisional identification was recently supported by comparison of the electron-spin-polarized EPR K-band spectrum of $P\text{-}700^+A_1^-$ with that of $P\text{-}870^+Q^-$ in iron-depleted bacterial reaction centers (Peterson et al., 1987). Because of the apparently exclusive localization of phylloquinone in PSI (Takahashi et al., 1985; Schoeder & Lockau, 1986), they suggested that phylloquinone may be the acceptor A_1.

Evidence for the participation of phylloquinone (vitamin K_1) on the reducing side of PSI was provided by Brettel et al. (1986), following a detailed analysis of absorbance transients in the UV at low temperature, and from quinone-depletion and biochemical reconstitution studies using phylloquinone (Biggins & Mathis, 1988; Itoh & Iwaki, 1989; Iwaki & Itoh, 1989, 1990).

Despite some anomalies regarding the EPR behavior of phylloquinone-depleted PSI preparations that still show electron transfer to terminal FeS centers at cryogenic temperatures (Sētif et al., 1987; Biggins et al., 1989), the functional role of phylloquinone in mediating electron transfer

† This work was supported by the Competitive Research Grants Office of the USDA (88-37130-4135) and the National Science Foundation (DMB-86-03586).

[1] Abbreviations: PSI, photosystem I; PSII, photosystem II; BPh^-, reduced bacteriopheophytin; Ph^-, reduced pheophytin; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

0006-2960/90/0429-7259$02.50/0

reactions between A_0^- and subsequent FeS redox centers has become generally accepted.

Recently, Iwaki and Itoh (1989, 1990) examined the interaction of a large number of benzo-, naphtho-, and anthraquinones with ether-extracted PSI preparations and reported that all species suppressed the recombination between P-700$^+$ and A_0^- and the decay of triplet P-700. On the basis of the observed kinetics for P-700$^+$ reduction following flash activation, they concluded that the quinones that promoted a 10–100-ms decay resulted in restoration of electron flow to F_x and then to F_AF_B. Effective quinones included phylloquinone, 9,10-anthraquinone, 2-methyl-9,10-anthraquinone, and 1-amino-9,10-anthraquinone. They also concluded that the quinone specificity of the site is not strict and that neither the naphthoquinone ring nor the phytyl tail is essential for the ability to function as A_1, although the latter contributed to tighter binding of the quinone to the A_1 site.

These observations are not in agreement with our original report on reconstitution of solvent-extracted PSI, where the interaction of phylloquinone was compared with that of vitamin K_3 (Biggins & Mathis, 1988). Unlike phylloquinone, which fully reconstituted PSI electron transfer and NADP$^+$ photoreduction, vitamin K_3 would only act as an exogenous electron acceptor and would not restore NADP$^+$ photoreduction. We therefore suggested that the phytyl tail in position 3 of the naphthoquinone head of phylloquinone was necessary to provide interaction of the quinone with the acceptor site in the core of PSI to promote electron transfer from A_0^- to F_x.

This report addresses this controversy directly by investigating the effect of selected benzo-, naphtho-, and anthraquinones at room temperature on the turnover of terminal FeS centers (P-430) in phylloquinone-depleted PSI. We confirm that the anthraquinones reported by Iwaki and Itoh (1989) act as exogenous electron acceptors, but in contrast we find that they do not donate to F_x and beyond to F_AF_B. In addition, our results suggest that, for 1,4-naphthoquinones, a hydrophobic side chain of C_{10} or longer appears to be necessary to promote electron donation from the quinone to F_x and restoration of complete electron transfer to terminal FeS centers.

Experimental Procedures

Biological Preparations. Spinach D-144 preparations were isolated according to Anderson and Boardman (1966), with high-grade digitonin obtained from Gallard-Schlesinger Chemical Mfg. Corp., NY. The PSI pellets were washed twice with deionized water, lyophilized, and stored in evacuated tubes at −80 °C. Phylloquinone was removed from the PSI by solvent extraction, which was performed as described previously with 100 mL of hexane containing 0.3% methanol/20 mg of PSI (Biggins & Mathis, 1988). The extracted PSI was then rehydrated in 0.05 M Tris-HCl, pH 8.0, containing 0.2% Triton X-100. After 30 min at 5 °C, the preparation was centrifuged at 10000*g* for 20 min to remove insoluble material.

Reconstitution Protocol. The following quinones were obtained from Aldrich Chemical Co.: 2,3,5,6-tetramethyl-1,4-benzoquinone; 2-methyl-1,4-naphthoquinone; 2,3-dimethyl-1,4-naphthoquinone; 2-methyl-3-phytyl-1,4-naphthoquinone; 9,10-anthraquinone; 1-amino-9,10-anthraquinone; and 2-ethyl-9,10-anthraquinone. Ubiquinone-30 and 2-methyl-3-(isoprenyl)$_4$-1,4-naphthoquinone were obtained from Sigma Chemical Co., and 3-decyl-1,4-naphthoquinone, 2-methyl-3-decyl-1,4-naphthoquinone, and 2-methyl-3-(isoprenyl)$_2$-1,4-naphthoquinone were kindly provided by Dr. Peter Rich (Glynn Research Institute, Bodmin, U.K.). Stock solutions of quinones (10 mM) were prepared in either methanol or dimethyl sulfoxide. Reconstitution of the extracted PSI preparation was by direct addition of saturating concentrations of the quinone (Biggins & Mathis, 1988) and incubation overnight at 5 °C in the dark. The long incubation time was chosen to insure adequate partitioning of the quinone between the solvent and the PSI (Biggins et al., 1990).

Flash Absorbance Spectrophotometry. Flash-induced absorbance transients were detected at 700 nm as described previously (Biggins et al., 1989) and in the wavelength range 400–500 nm by using an EMI 9558 photomultiplier. The selected bandwidth of the detection system was DC to 3 kHz. The transients were averaged by using a 125-MHz digital oscilloscope (Model 9400, LeCroy, Chestnut Ridge, NY). Flash activation was via a Q-switched, frequency-doubled Nd/YAG laser (Model DCR 11, Spectra Physics, Mountain View, CA).

Results

Restoration of P-430 Turnover in Quinone-Depleted PSI by Use of Phylloquinone. The experimental strategy adopted for determination of the restoration of electron transfer to terminal FeS centers at room temperature was to measure P-430 turnover optically by kinetic analysis of flash transients obtained in the 400–500-nm range. As shown originally by Ke and co-workers (Hiyama & Ke, 1971; Ke, 1972, 1973), the P-430 contribution can be estimated by a comparison of the flash-induced transient of PSI undergoing P-700$^+$/P-430$^-$ recombination with that of PSI poised for P-700$^+$ photoaccumulation in the presence of a low-potential secondary acceptor. In the blue region of the spectrum, the absorbance signal in the recombination reaction results from contributions from both P-700$^+$ and P-430$^-$. The absorbance due to P-700$^+$ alone can be determined in PSI samples supplemented with methyl viologen because viologens interact efficiently with P-430$^-$ and the reduced semiquinone reacts with O_2 but not P-700$^+$. Therefore, on a slow time scale (>1 ms) the flash transient represents only the P-700 bleaching and subsequent reduction of P-700$^+$ by ascorbate/TMPD (Ke, 1973).

Figure 1 shows flash-induced absorbance transients obtained at 430 and 700 nm for control, solvent-extracted, and phylloquinone-reconstituted preparations of PSI. The recombination reaction between P-700$^+$ and P-430$^-$ is promoted by poising the preparations with ascorbate/TMPD (condition A). The P-700$^+$ photoaccumulation and subsequent chemical reduction is induced by including methyl viologen in the reaction mixture (condition B).

In the recombination reaction (condition A), the control PSI showed a half-time of 50–80 ms rather than the usual 30–45-ms half-time (Hiyama & Ke, 1971). This difference was most likely due to the extensive physical handling of the PSI preparation (washing, lyophylization, and rehydration) that was necessary for removal of the endogenous phylloquinone by extraction with the organic solvents. Addition of methyl viologen to the control resulted in a decrease of ΔA at 430 nm because of elimination of the contribution due to P-430$^-$ from the signal and, as expected, a much slower decay rate due to the reduction of P-700$^+$ by ascorbate/TMPD. Flash-induced transients for the control PSI samples measured at 700 nm showed the same ΔA in conditions A and B because only P-700$^+$ absorbs at this wavelength, but the decay kinetics for samples in each condition were identical with those observed at 430 nm.

Transients for samples in these protocols were then acquired for hexane/methanol-extracted PSI and for the extracted PSI reconstituted with phylloquinone. The flash-induced transients in Figure 1 for the extracted PSI preparation show no significant absorbance signals because of the slow time response

Table I: Quinone Structural/Redox Potential Requirements for Reconstitution of PSI Electron Transfer to Terminal FeS Clusters (P_{430})

quinone	half-reduction potential Q-Q^- (mV)	flash transients at 430 nm[a] donor system[b] ΔA ($\times 10^{-3}$)	donor system[b] $t_{1/2}$ (ms)	donor system + acceptor[c] ΔA ($\times 10^{-3}$)	donor system + acceptor[c] $t_{1/2}$ (ms)	electron transfer to P-430
1,4-naphthoquinones						
head substitutions (25 μm)						
unsubstituted	−581[d]	3.9	239	3.8	257	−
2-methyl (vitamin K_3)	−650[d]	4.5	245	4.4	230	−
2,3-dimethyl	−746[d]	4.1	251	4.0	263	−
tail substitutions (10 μm)						
3-decyl	−680[e]	4.0	260	3.9	269	−
2-methyl, 3-decyl	−730[d]	3.7	83	2.9	260	+
2-methyl, 3-phytyl (vitamin K_1)	−710[f]	4.5	69	3.5	258	+
2-methyl, 3-(isoprenyl)$_2$	−709[d]	4.0	68	3.3	264	+
2-methyl, 3-(isoprenyl)$_4$	−710[e]	4.1	71	3.3	267	+
1,4-benzoquinones (25 μm)						
2,3,5,6-tetramethyl	−750[d,g]	3.9	294	3.8	289	−
ubiquinone-30	−610[e]	3.8	283	3.9	281	−
9,10-anthraquinones (25 μm)						
unsubstituted	−830[g]	3.8	170	4.0	250	−
1-amino	−940[g]	4.3	185	4.4	235	−
2-ethyl	−850[g]	3.7	163	3.8	243	−
control, not extracted		6.32	53	4.6	234	+

[a] Chlorophyll concentrations: 8 μg/mL for the control, 12 μg/mL for the reconstituted samples. [b] Ascorbate/TMPD. [c] Ascorbate/TMPD plus viologen. [d] Prince et al., 1983. [e] Estimated. [f] Iwaki & Itoh, 1989. [g] Woodbury et al., 1986.

of the detection system selected for use in this study. We showed previously that with instrumentation of nanosecond time resolution the flash transients at 820 nm revealed a ca. 30-ns recombination between P-700$^+$ and A_0^- and a microsecond-range decay of triplet P-700 formed from the primary radical pair (Biggins & Mathis, 1988). The fast kinetic phases were replaced by slower relaxations resembling those of unextracted PS1 when the extracted PSI was reconstituted with phylloquinone. The data in Figure 1 now confirm and extend those results. The preparation reconstituted with 5 μM phylloquinone showed flash transients at 430 and 700 nm that were very similar to those of the unextracted control discussed above with respect to relative signal amplitudes and decay kinetics. The transients at 430 nm (conditions A and B) are, therefore, suggestive of the participation of P-430 in the process of charge stabilization in the preparation reconstituted with phylloquinone.

Confirmation that the absorbance transients measured at 430 nm were indeed diagnostic for the turnover of P-430 in the reconstituted sample is presented in the point difference spectra in Figure 2. The spectrum of P-700$^+$ minus P-700, condition B (●), was subtracted from the total changes (condition A, not shown) to give a difference spectrum of P-430$^-$ minus P-430 (○)(Hiyama & Ke, 1971; Ke, 1972). This difference spectrum is taken as unequivocal evidence that the flash transients at 430 nm in conditions A and B do measure the turnover of the terminal FeS centers and that phylloquinone restores the entire electron transfer sequence on the reducing side of PSI. These data confirm our earlier reports that phylloquinone restores NADP$^+$ photoreduction in solvent-extracted PS1 preparations (Biggins & Mathis, 1988) and the observed correlation between phylloquinone concentration and capacity for NADP$^+$ photoreduction in PS1 preparations depleted of endogenous quinone by UV destruction (Biggins et al., 1989). The observations are also consistent with the report of Itoh and Iwaki (1989), who constructed a similar P-430 difference spectrum from fast (0.5 ms) and extrapolated slow (5 ms) decay components of flash transients from phylloquinone-reconstituted ether-extracted PSI in the presence of benzylviologen.

Interaction of Other Quinones with Extracted PSI Preparations. Selected benzoquinones, naphthoquinones, and anthraquinones were then studied to gain insight regarding the structural/redox properties necessary for successful restoration of the PSI electron transfer sequence. Table I lists selected quinones tested, which include both head and alkyl tail substituents of 1,4-naphthoquinone and substituted 1,4-benzo-

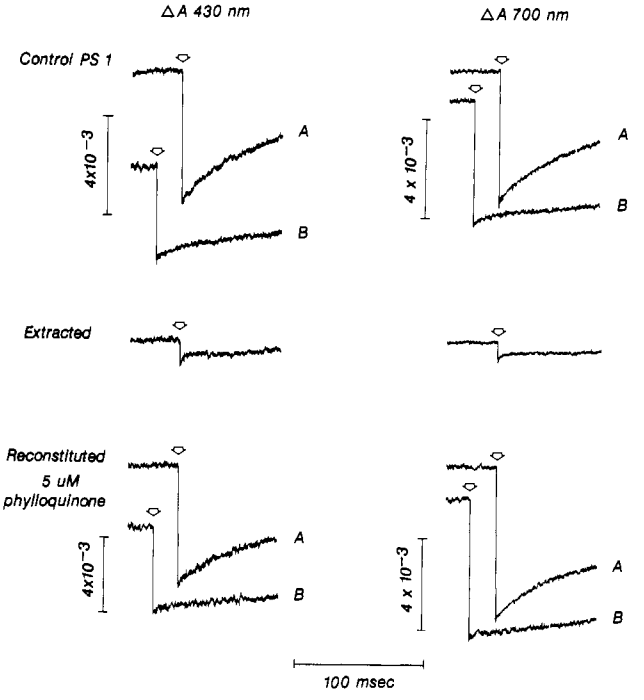

FIGURE 1: Flash-induced absorbance transients at 430 and 700 nm in D-144 preparations from spinach. The preparations were poised with the donors 2 mM sodium ascorbate plus 25 μM TMPD (A) and supplemented with the secondary acceptor 50 μM methyl viologen (B). The control PSI was rehydrated PSI after washing and lyophylization, extracted was PSI extracted with hexane/methanol, and reconstituted was solvent-extracted PSI reconstituted with 5 μM phylloquinone. Transients at 430 and 700 nm were recorded with the same samples in each treatment. Chlorophyll concentrations were as follows: control, 12 μg/mL; extracted and reconstituted, 8 μg/mL. The optical path length was 10 mm, and transients were recorded at room temperature. The downward arrows denote firing of the laser flash. The pairs of transients are offset for clarity. The transients at 430 nm were the average of 50 flashes, and those at 700 nm were the average of 20 flashes.

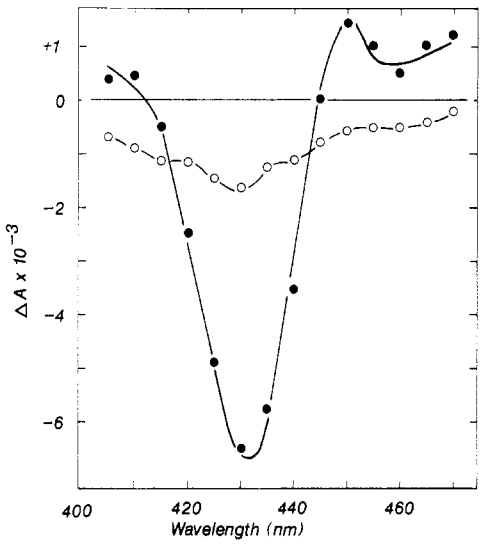

FIGURE 2: Point by point difference spectra for extracted PSI reconstituted with 5 μM phylloquinone. The spectrum of P-700$^+$ minus P-700 (●) was obtained from flash transients of samples containing 2 mM sodium ascorbate, 25 μM TMPD, and 50 μM methyl viologen (i.e., condition B in Figure 1). The spectrum P-430$^-$ minus P-430 (○) was obtained by subtraction of the P-700$^+$ difference spectrum from that of a sample with no methyl viologen (i.e., condition A in Figure 1). Other conditions were as in Figure 1. The difference spectra were assembled from flash transients obtained from four preparations of reconstituted PSI. The experimental error was 4×10^{-4} $\Delta A/2$ nm.

quinones and 9,10-anthraquinones of low reduction potential.

With respect to the flash transient assay for P-430 turnover as outlined above, the majority of the quinones tested suppressed the nanosecond-range recombination between P-700$^+$ and A_0^- but did not restore P-430 turnover. The flash transients determined at 430 nm from PSI reconstituted with these quinones in the presence of donor (condition A) or when supplemented with methyl viologen (condition B) were identical. The signal amplitudes were the same and the recoveries were slow (ca 280 ms). Similar transients were observed at 700 nm (not shown), and it can be concluded that the signal was due to P-700$^+$ photoaccumulation followed by reduction by the donor ascorbate/TMPD. This interpretation was confirmed by point difference spectra conducted as for Figure 2. The difference spectra (not shown) indicated no contribution from P-430. Therefore, all these quinones act as exogenous electron acceptors, indicating that the A_0^- donation site is relatively unspecific for the acceptor. Recently we have also shown that nonquinonoids such as anthrone and fluorenone (Warncke & Dutton, 1990) are also very effective acceptors (manuscript in preparation).

In contrast, only three quinones, in addition to phylloquinone, were found to restore P-430 turnover. In these cases [2-methyl-3-decyl-, 2-methyl-3-(isoprenyl)$_2$-, and 2-methyl-3-(isoprenyl)$_4$-1,4-naphthoquinones], the flash transients observed in the presence of donor revealed relaxation kinetics diagnostic for the P-700$^+$ P-430$^-$ recombination reaction (71–83 ms), and the addition of methyl viologen resulted in a ca. 20% decrease in amplitude of the transient and conversion to much slower kinetics. Point difference spectra (not shown) confirmed the turnover of P-430, indicating successful restoration of the entire electron transfer sequence. It is notable that these quinones are structurally similar to phylloquinone in that they are 1,4-naphthoquinones with a methyl group at position 2 and an alkyl tail at position 3.

Discussion

The results presented here confirm our original report that phylloquinone fully reconstitutes the electron transfer sequence of PSI preparations depleted of quinone by organic solvent extraction (Biggins & Mathis, 1988). These findings were based upon kinetic analysis of flash transients obtained at room temperature in time ranges from nanoseconds to milliseconds. The optical data corroborated biochemical measurements where we showed that phylloquinone, but not vitamin K_3, was able to reconstitute the photoreduction of NADP$^+$. The demonstration in the present report that the optical signal due to the terminal FeS centers, F_AF_B (P-430), was lost following quinone extraction and restored upon reconstitution with phylloquinone extends the earlier results and reinforces the postulate that the redox center A_1 is phylloquinone.

When first reported, the extraction/reconstitution data were questioned because the same quinone-depleted PSI preparations unexpectedly showed the photoreduction of the terminal FeS centers when examined by EPR at cryogenic temperatures (Sétif et al., 1987; Biggins et al., 1989). We addressed the anomaly, and alternate models for PSI electron transfer were suggested (Sétif et al., 1987; Biggins & Mathis, 1988). These included the possibility of a bypass of the A_1 site induced by solvent extraction and freezing, or alternate pathways. Recently, Ikegami et al. (1990) reported that an ether-extracted preparation of PSI from the cyanobacterium *Synechococcus* does not show the photoreduction of terminal FeS centers at 20 K, as would be expected if quinone is a component of the electron transfer pathway. They also showed that the FeS centers were photoreduced in the extracted PSI preparation following reconstitution with both phylloquinone and vitamin K_3.

The quinone replacement studies presented here complement the investigation of Iwaki and Itoh (1989), and we concur that a wide variety of quinones can act as exogenous electron acceptors and oxidize the primary photooxidant, A_0^-. On the other hand, the observations reported here on the effectiveness of various quinone substituents on restoration of electron transfer from the A_1 site to terminal FeS centers are at total variance with the conclusions of Iwaki and Itoh (1989, 1990). These investigators categorized a large number of substituted benzo-, naphtho-, and anthraquinones by their ability to react with quinone-depleted PSI preparations and reported a correlation between their kinetic pattern and the estimated E_m values of the semiquinone$^{\bullet-}$/semiquinone couple. They reported that one category of quinones (E_m −800 to −1000 mV) promoted a $t_{1/2}$ = 10–100 ms decay rate. This category included anthraquinone, 2-methyl-9,10-anthraquinone, and 1-amino-9,10-anthraquinone in addition to phylloquinone and vitamin K_3. They concluded that these quinones functioned as the redox center A_1 and mediated full electron flow from A_0^- to F_x and then to F_B and F_A. In the present study, using a more exacting criterion for reconstitution, none of these quinones were observed to restore electron transfer to terminal FeS centers except phylloquinone. This indicates that consideration of the kinetics of the flash transient induced by a potential replacement quinone alone is not a sufficiently stringent criterion to be used as an indicator of reconstitution of complete electron transfer.

A result in Table I also shows that vitamin K_3 (2-methyl-1,4-naphthoquinone) does not promote P-430 turnover, and this confirms our original report that this naphthoquinone does not restore NADP$^+$ photoreduction (Biggins & Mathis, 1988).

The data presented in Table I show that only three naphthoquinones, in addition to phylloquinone, restore electron flow to P-430; namely, 2-methyl-3-decyl-1,4-naphthoquinone, 2-methyl-3-(isoprenyl)$_2$-1,4-naphthoquinone, and 2-methyl-3-

(isoprenyl)$_4$-1,4-naphthoquinone. These naphthoquinones are structurally similar to phylloquinone in that they all have a methyl group at position 2 and an alkyl side chain at position 3 on the quinone head. The implication from this result is that a hydrophobic tail on the A_1 replacement quinone appears to be required to facilitate the reduction of F_x by the semiquinone$^{\bullet-}$. We have previously suggested (Biggins & Mathis, 1988) that the 3-phytyl tail on phylloquinone is necessary to provide the interaction between the quinone and the A_1 binding domain, resulting in the correct in situ E_m for the electron transfer intermediate. This suggestion is further supported in this study because 2,3-dimethyl-1,4-naphthoquinone (E_m = −746 mV) was expected to react thermodynamically like 2-methyl-3-decyl-1,4-naphthoquinone (E_m = −730 mV) but failed to restore the electron transfer sequence, whereas the quinone with the 3-alkyl tail was fully effective in reconstitution.

An additional parameter for consideration in the reconstitution of PSI activity is the partition coefficient of the replacement quinone (P. Rich, personal communication). In this respect, it is of interest to note that 3-decyl-1,4-naphthoquinone was expected to partition similarly to the other naphthoquinones with hydrophobic tails at position 3, but in this instance no P-430 turnover was observed. As the reduction potential of 3-decyl-1,4-naphthoquinone (E_m = 680 mV) is ca. 50 mV more positive than the naphthoquinones with a position 2 substituent (Rich & Bendall, 1980), it is possible that the in situ E_m was too high to permit action as A_1. Alternatively, the position 2 methyl group may be a structural feature needed for the biological reconstitution.

These conclusions contrast with those of Iwaki and Itoh (1989), who claim that the 3-phytyl tail of phylloquinone is not essential and, by analogy, that the A_1 quinone binding site is similar to the bacterial reaction center Q_A site. For the Q_A site it has been shown conclusively that hydrophobic tails on replacement quinones exert a minor effect (Gunner et al., 1986; Warncke et al., 1987). Although it might be anticipated that the mechanism of electron transfer between A_0^- and A_1 may be similar to that between bacterial BPh^- and Q_A (Gunner et al., 1986), or PSII Ph^- and Q_A of higher plants (Crofts & Wraight, 1983), the electron transfer from A_1^- to F_x appears to have different constraints. The redox center F_x is a 4Fe–4S cluster whose cysteine ligands are contributed from the two polypeptides of the PSI core heterodimer (Parrett et al., 1989), whereas in bacterial reaction centers and PSII, the primary quinone, Q_A^-, interacts with a secondary quinone, Q_B. Although there is a discrete binding site for the secondary quinone, Q_B, the redox intermediate is exchangeable and facilitates electron transfer to a bulk quinone pool. The interaction between Q_A^- and Q_B in bacterial reaction centers and PSII is, therefore, almost certain to be different from the electron transfer between A_1^- and F_x in PS1. It is conceivable that a specific orientation of the A_1 quinone is required to promote efficient PSI electron transfer and that this alignment in the A_1 binding site may be effected by interaction of the 3-alkyl side chain with a hydrophobic domain in the PSI core.

ACKNOWLEDGMENTS

I wish to thank Dr. Peter Rich for his generosity in furnishing samples of various substituted quinones and Dr. S. I. Beale for helpful advice and suggestions concerning the manuscript.

Registry No. 1,4-Naphthoquinone, 130-15-4; 2-methyl-1,4-naphthoquinone, 58-27-5; 2,3-dimethyl-1,4-naphthoquinone, 2197-57-1; 3-decyl-1,4-naphthoquinone, 41245-48-1; 2-methyl-3-decyl-1,4-naphthoquinone, 117157-41-2; 2-methyl-3-phytyl-1,4-naphthoquinone, 84-80-0; 2-methyl-3-(isoprenyl)$_2$-1,4-naphthoquinone, 1163-13-9; 2-methyl-3-(isoprenyl)$_4$-1,4-naphthoquinone, 6041-00-5; 2,3,5,6-tetramethyl-1,4-naphthoquinone, 127999-43-3; ubiquinone 30, 1065-31-2; 9,10-anthraquinone, 84-65-1; 1-amino-9,10-anthraquinone, 82-45-1; 2-ethyl-9,10-anthraquinone, 84-51-5.

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A Shared Internal Threonine–Glutamic Acid–Threonine–Proline Repeat Defines a Family of *Dictyostelium discoideum* Spore Germination Specific Proteins†

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Received February 27, 1990; Revised Manuscript Received April 30, 1990

ABSTRACT: A cDNA denoted pRK270 hybridizes to two mRNA species in RNA blots. The mRNAs specific to this clone are not expressed during vegetative growth and multicellular development. They are, however, found predominantly during early stages of spore germination, suggesting that their synthesis is rapidly and coordinately turned on during germination. Two different cDNAs named 270-6 and 270-11 were isolated, representing the two mRNAs. DNA blot analysis shows that 270 is a multigene family. Four genes were isolated from *Dictyostelium* genomic libraries and sequenced. The putative proteins coded for by these genes are about 51 000, 55 000, 76 000, and 100 000 Da. Two of the genes are expressed during spore germination while transcripts for the other two are not present during spore germination, vegetative growth, or the stages of multicellular development studied. The cDNAs and genes code for deduced proteins that possess a very unusual internal amino acid repeat comprised of the tetrapeptide threonine–glutamic acid–threonine–proline. The other portions of the proteins have no homology among themselves. The 270-6 protein shows excellent identity with avocado (*Persea americana*) cellulase, indicating that it may function as an *endo*-(1,4)-β-D-glucanase.

Dictyostelium discoideum is a favorable organism for studying the macromolecular events coincident with and necessary for eukaryotic development. The life cycle is short, and development can be synchronized and separated from vegetative growth (Sussman & Brackenbury, 1976). Consequently, biochemical and morphological changes that occur can be correlated, and developmentally critical events may be revealed. In addition, the organism has a small genome, which makes its analysis and selection of specific genes easier than in more complex eukaryotes.

One of the central problems of developmental biology is to determine the nature of the mechanisms that control the activation and expression of developmentally critical genes. For this purpose it is first necessary to isolate and identify these genes. In previously reported work we identified proteins that are developmentally regulated during spore germination (Dowbenko & Ennis, 1980; Giorda & Ennis, 1987; Giri & Ennis, 1978; Kelly et al., 1983). Spore germination in *D. discoideum*, similar to other stages in slime mold development, is accompanied by developmentally regulated changes in both protein and mRNA synthesis (Dowbenko & Ennis, 1980; Giri & Ennis, 1978; Kelly et al., 1983), and this makes the process a favorable one for developmental studies. A number of cDNA clones were isolated representing mRNA that is present only in spores and/or during spore germination, and these cDNAs have been used to isolate specific genomic sequences. One developmentally regulated cDNA on which the present study focuses, named pRK270, hybridized to mRNA present almost exclusively during early spore germination. This mRNA did not accumulate during growth or multicellular development and was present in very low concentration in dormant spores (Kelly et al., 1983; Shaw et al., 1986). pRK270 is a member of a multigene family containing four different genes, and we have isolated and sequenced all of them. A common feature of the deduced protein sequences is an internally located repeat of the tetrapeptide threonine–glutamic acid–threonine–proline.

Experimental Procedures

Previously Described Methods. All methods involving growth of *D. discoideum*, preparation of spores and analysis of spore germination, isolation of RNA and DNA, RNA and DNA blot analysis, labeling of DNA probes, DNA sequencing, and sources of materials were those described (Giorda & Ennis, 1987).

Preparation of cDNA and Genomic Libraries. cDNA libraries were prepared from poly(A)$^+$-selected 1.5-h germination RNA. The libraries were constructed in λgt10 as described by the manufacturer of the cloning kit (Amersham, Arlington Heights, IL). The two cDNAs isolated were named λTO270-6 (which contains the sequences of the original pRK270 clone but is longer) and λTO270-11.

A genomic library of sheared AX3 DNA, to which were added *Eco*RI linkers, was cloned in λZAP bacteriophage by Strategene (San Diego, CA). Most clones were isolated from this library. Clone 270-P was isolated from another library constructed by using approximately 6-kb *Eco*RI fragments inserted into λgt10. One small clone containing the 270-11 intron (270-PCR) was constructed by using the polymerase

† The nucleic acid sequences in this paper have been submitted to GenBank under Accession Number J02916.
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