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Probing the Structure of Photosystem II with Amines and Phenylhydrazine*

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Photosynthetic oxygen evolution is catalyzed at the manganese-containing active site of photosystem II (PSII). Amines are analogs of substrate water and inhibitors of oxygen evolution. Recently, the covalent incorporation of ^{14}C from [^{14}C]methylamine and benzylamine into PSII subunits has been demonstrated (Ouellette, A. J. A., Anderson, L. B., and Barry, B. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2204–2209). To obtain more information concerning these labeling reactions, *t*-[^{14}C]butylamine and phenylhydrazine were employed as probes. Neither compound can be oxidized by a transamination or addition/elimination mechanism, but both can react with activated carbonyl groups, produced as a result of posttranslational modification of amino acid residues, to give amine-derived adducts. ^{14}C incorporation into the PSII subunits D2/D1 and CP47 was obtained upon treatment of PSII with either *t*-[^{14}C]butylamine or [^{14}C]phenylhydrazine. For *t*-butylamine and methylamine, the amount of labeling increased when PSII was treated with denaturing agents. Labeling of CP47, D2, and D1 with methylamine and phenylhydrazine approached a one-to-one stoichiometry, assuming that D2 and D1 each have one binding site. Evidence was obtained suggesting that reductive stabilization and/or access are modulated by PSII light reactions. These results support the proposal that PSII subunits D2, D1, and CP47 contain quinocofactors and that access to these sites is sterically limited.

Photosystem II (PSII),[1] a membrane-bound protein complex, catalyzes the light-driven oxidation of water and the reduction of plastoquinone. PSII consists of hydrophobic and extrinsic subunits and contains chlorophyll, plastoquinone, manganese, and several other bound cofactors. Water oxidation occurs at a manganese-containing catalytic site located on the lumenal side of the membrane. After photoexcitation, the primary chlorophyll donor, P_{680}, transfers an electron from its excited state to a pheophytin molecule. Pheophytin transfers the electron to a quinone acceptor molecule, Q_A, which in turn reduces a second quinone acceptor, Q_B (reviewed in Refs. 1 and 2). PSII contains two well characterized redox-active tyrosines, Z and D (3, 4). Z, an intermediate electron carrier between P_{680} and the manganese cluster, reduces oxidized P_{680} (5). D forms a stable radical and has no known role in water oxidation (6). Oxidation of a third, posttranslationally modified tyrosine, M, occurs in PSII site-directed mutants (6–9).

Primary amines are known to be substrate analogs and inhibitors of oxygen evolution (10–12). Recently, the covalent incorporation of ^{14}C-labeled primary amines into PSII subunits has been observed under reducing conditions (13). Evidence for oxidation of [^{14}C]benzylamine by PSII was obtained by detection and quantitation of [^{14}C]benzaldehyde (13). Because PSII exhibits this amine oxidation activity and an aldehyde product has been observed, covalent incorporation of ^{14}C from labeled primary amines into PSII subunits could be caused by two possible reactions (13). In the first possible reaction, addition of reductant traps a Schiff base complex of substrate (^{14}C-labeled amine) and an amino acid side chain that has been posttranslationally modified to contain an activated carbonyl group (13, 14, 15). In the second possible reaction, addition of reductant traps a Schiff base complex of product (^{14}C-labeled aldehyde) and the amino group of a lysine side chain (13). When formaldehyde is produced, this reaction produces mono- and dimethylated lysines under reducing conditions (Ref. 16 and references therein). Other types of modification reactions with formaldehyde are also possible (16).

To distinguish between these possibilities, we have employed ^{14}C-labeled compounds, which would not be expected to form a [^{14}C]aldehyde product through a transamination reaction but can bind to posttranslationally modified amino acid residues, containing activated carbonyl groups. For a review of previously discovered cofactors of this type, see Ref. 17. Our SDS-PAGE results provide chemical evidence for quinocofactors in PSII. The significance of these findings is discussed.

EXPERIMENTAL PROCEDURES

Reagents—Purified bovine serum AO was a generous gift from Prof. J. Klinman and Dr. S. Wang (13). Fatty acid-free BSA was purchased from Sigma. [^{14}C]Methylamine (specific activity, 57 mCi/mmol; 1 Ci = 37 GBq) and [^{14}C]BSA (specific activity, 12.5 μCi/mg) were obtained from Amersham Pharmacia Biotech. [^{14}C]Phenylhydrazine (specific activity, 2.65 mCi/mmol) was obtained from California Bionuclear Corp. (Los Angeles, CA). *t*-[^{14}C]Butylamine (specific activity, 55 mCi/mmol) was purchased from Morevek Biochemicals, Inc. (Brea, CA). A mono-

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[1] The abbreviations used are: PSII, photosystem II; PSII-1, spinach PSII membranes; PSII-2, spinach PSII membranes lacking the 24- and 18-kDa extrinsic proteins; PSII-3, spinach PSII membranes lacking a functional manganese cluster, the manganese stabilizing protein, and other extrinsic subunits; BSA, bovine serum albumin; AO, amine oxidase; chl, chlorophyll; DEN+MA, [^{14}C]methylamine present during sample denaturation, but not before denaturation; DEN//MA, [^{14}C]methylamine present after sample denaturation, but not before or during denaturation; DTT, dithiothreitol; MA, methylamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; μE, microeinstein(s); MSP, manganese stabilizing protein; PAGE, polyacrylamide gel electrophoresis; R-1st, chemical reductant added prior to sample denaturation; R-NO, no chemical reductant present in sample buffer; R-2nd, chemical reductant added after sample denaturation; SIM, chemical reductant, urea, and SDS added to sample simultaneously; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing Tween-20.

clonal antibody against spinach CP47 was a generous gift from Prof. T. Bricker (18).

PSII Purification—Chlorophyll assays and O_2 evolution assays were performed by methods described previously (19). PSII membranes (PSII-1) were isolated from spinach by the method of Berthold *et al.* (20) with the following modifications (as suggested by Refs. 21 and 22 and references therein). Spinach was homogenized at 4 °C in a medium containing 400 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 50 mM HEPES-NaOH, pH 7.5, and 2 mg/ml BSA. After filtration through four layers of cheesecloth, the homogenate was centrifuged at 6000 × *g* for 10 min. The pellet was resuspended in wash buffer (150 mM NaCl, 4 mM $MgCl_2$, and 20 mM MES-NaOH, pH 6.0) and centrifuged at 12,000 × *g* for 10 min. The pellet was then resuspended in Triton buffer (15 mM NaCl, 5 mM $MgCl_2$ and 50 mM MES-NaOH, pH 6.0). A Triton X-100 stock solution was made up at 25% (w/v) in Triton buffer. This stock solution was added dropwise to the resuspended pellet during shaking on ice in the dark. The final chlorophyll concentration was 2 mg of chl/ml, and the ratio of Triton X-100 to chl was 2.5 mg of Triton/mg of chl. Shaking on ice was continued in the dark for 30 min. The suspension was immediately centrifuged at 47,800 × *g* for 30 min. The pellet was resuspended in SMN buffer (400 mM sucrose, 15 mM NaCl, and 50 mM MES-NaOH, pH 6.0) and centrifuged at 47,800 × *g* for 30 min. Finally, PSII membranes were resuspended in SMN buffer to a final concentration of 3–5 mg chl/ml and stored at −70 °C. The steady-state rate of oxygen evolution was in the range of 800–1000 μmol of O_2/mg of chl-h.

PSII-2 samples were generated by salt washing to remove the 24- and 18-kDa proteins and other extrinsic subunits (23). PSII-3 samples were generated by depletion of manganese, MSP, and other extrinsic subunits (13). All PSII samples were chloride-depleted by resuspending three times in a buffer containing 400 mM sucrose and 100 mM HEPES-NaOH, pH 7.5.

Amine and Phenylhydrazine Binding Assays—Binding experiments were performed under room light as described previously (13). The incubation time, prior to protein denaturation, was 4 h for *t*-butylamine, 4 h or 30 min for methylamine, and 30 min for phenylhydrazine. In some experiments, methylamine was added during or after protein denaturation. The protein sample was denatured by the addition of denaturing buffer, giving final concentrations of 2.7 M urea, 2.1% SDS, and 52 mM Na_2CO_3. In some experiments, DTT was added as an exogenous reductant to give a final concentration of 62 mM. The order of addition of buffer components was varied in some experiments; each addition was followed by incubation at room temperature as shown in Fig. 1. Experiments carried out in the light were performed under 4 μE/m^2-s of fluorescent bulb-derived room illumination. Some experiments were carried out in the dark; in this case, incubations, additions, and SDS-PAGE were all performed in light intensities less than or equal to 0.7 μE/m^2-s, and the available illumination was green-filtered.

SDS-PAGE Analyses—Samples were subjected to SDS-PAGE according to the modified Neville method (24). Gels were run in duplicate. One gel was stained with Coomassie Brilliant Blue R (25). The contents of the other gel were transferred to Immobilon P^{SQ} transfer membranes (Millipore Corporation, Bedford, MA) using a TE70 Semi Phor semidry transfer unit (Hoeffer Scientific Instruments, San Francisco, CA). Transfer buffer, pH 8.8, contained 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% methanol. The amount of protein remaining in each gel lane after the transfer procedure was visualized by Coomassie staining. The percent protein transferred ranged from 63 to 95%. There was no observed, statistical correlation between the percentage protein transferred and the amount of ^{14}C detected (data not shown).

Phosphorimages were obtained by scanning phosphor screens with a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA). Immobilon membranes were exposed to the phosphor screens for 3–27 days. Personal Densitometer SI (Molecular Dynamics) was used to scan Coomassie-stained gels. Images were quantitated using Image Quant software (Molecular Dynamics). To correct for variations in background, the number of counts/pixel in the background was averaged from several positions in the image. This average background was subtracted from the number of counts/pixel retained by ^{14}C-labeled protein subunits. The number of counts/pixel, retained by protein subunits, was found to be linearly related to exposure time (data not shown). Data were then normalized to the number of counts/pixel for fatty acid-free BSA, which was run on each gel as a negative control. The number of counts/pixel retained by BSA, treated by methods 2 and 4 (Fig. 1), was averaged. Ratioing to BSA corrects for variation in background caused by nonspecific binding of ^{14}C-labeled compounds to proteins and for variations in exposure time. Finally, values were averaged over multiple experiments, and standard errors were calculated.

Western Assays—For Western analysis, Immobilon membranes were blocked with TBS (20 mM Trizma-NaOH, pH 7.4, and 137 mM NaCl) containing 5% low-fat dried milk for 2.5 h and incubated with a mouse monoclonal antibody to CP47. The incubation time was 1 h. The membrane was rinsed with TBS containing 0.1% Tween-20 (TBS-T), three times with 15-min incubations, and then twice with 5-min incubations. The incubation time in TBS-T with the peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch, Bar Harbor, ME) was 1 h. The membrane was washed again as described above. Chemilumenescence reagents (ECL, Amersham Pharmacia Biotech) and x-ray film (Kodak) were used for detection.

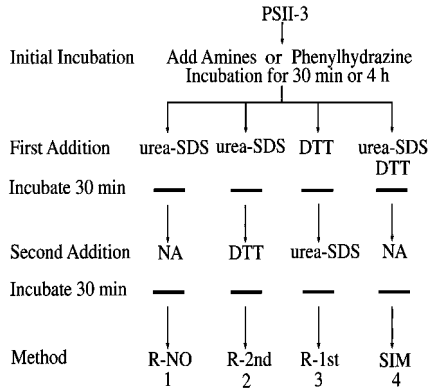

FIG. 1. **Protocol for order of addition experiment.** The chart describes the order in which buffer components were added to protein samples, which had been preincubated with 4 mM methylamine, 7 mM *t*-butylamine, or 56 μM phenylhydrazine. The free base concentrations for methylamine and phenylhydrazine were 2.8 μM, and the free base concentration for *t*-butylamine was 3.2 μM. *NA,* no addition.

RESULTS

Phosphorimaging Can be Used to Detect ^{14}C Labeling of PSII Subunits—We have previously shown that PSII-3, which lacks manganese, MSP, and other extrinsic subunits, incorporates ^{14}C from [^{14}C]benzylamine and [^{14}C]methylamine into the PSII subunits CP47 and D2/D1 (13). PSII-1, which retains manganese and these subunits, did not carry out these reactions. This difference was attributed to increased access to posttranslationally modified amino acids at the active site, which are normally inaccessible. This interpretation was supported by data showing that removal of the 18- and 24-kDa extrinsic subunits alone (generating PSII-2), is sufficient to allow ^{14}C labeling to occur, in the absence of Cl^- (13). These subunits are known to influence the access of reductants to the catalytic site (for example, see Ref. 26). In these experiments, SDS-PAGE and fluorography were used to visualize the labeled subunits (13).

The results of a similar experiment employing the more sensitive phosphorimaging method are presented in Fig. 2. In this binding assay, PSII-1, PSII-2, and PSII-3 were incubated for 4 h in the presence of [^{14}C]methylamine. Urea, SDS, and DTT were added simultaneously (method 4, Fig. 1) to the samples; these are the conditions employed in our previous studies (13). The samples were subjected to SDS-PAGE and transferred from the gel to Immobilon P^{SQ}. The resulting phosphorimage (Fig. 2) confirms that significant ^{14}C labeling of CP47 and D2/D1 subunits occurred in PSII-2 and PSII-3 samples under these conditions (*lanes 2* and *3*). ^{14}C incorporation into PSII-3 is increased by a factor of 2 when compared with the amount of ^{14}C incorporated into PSII-2 (Fig. 2, compare *lanes 2* and *3*) and increased by a factor of 12 when compared with the background amount of ^{14}C retained by PSII-1 (compare *lanes 1* and *3*). As a positive control, AO, which is known to contain a topaquinone quinocofactor (27), was also included in the experiment (Fig. 2, *lane 4*). The expected incorporation of ^{14}C into monomeric AO subunits was observed (13, 14). As a negative control, fatty acid-free BSA was employed (Fig. 2, *lane 5*). As

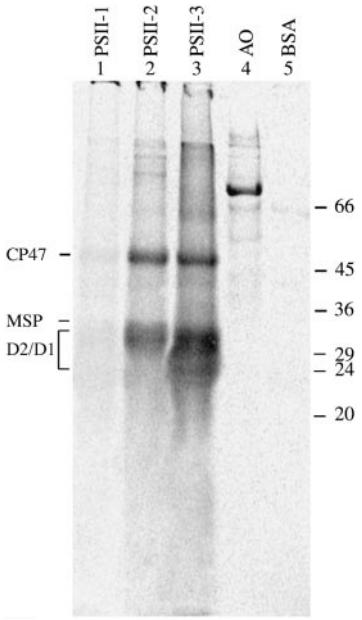

FIG. 2. **Methylamine binding to PSII and AO subunits.** Samples were incubated with 4 mM [^{14}C]methylamine-hydrochloride for 4 h, subjected to SDS-PAGE, and blotted onto Immobilon membranes. Method 4 (see Fig. 1) was employed. The phosphorimage is shown. *Lane 1,* PSII-1; *lane 2,* PSII-2; *lane 3,* PSII-3; *lane 4,* AO; *lane 5,* BSA. The electrophoretic migration of molecular mass standards (kDa) is indicated at the *right*.

expected, there was no significant incorporation of ^{14}C label into BSA.

The Order of Addition Affects ^{14}C Labeling from Methylamine—In Fig. 3, *A–C,* we present the results of an order of addition experiment (Fig. 1) employing methylamine. SDS-PAGE analysis was performed on PSII-3, which was treated by each of the four methods shown in Fig. 1. In each method, methylamine was added to PSII-3, and the sample was incubated for 30 min under room illumination. In Fig. 3, *A–C, lane 1,* urea and SDS (denaturants) were added to the methylamine-containing PSII-3 sample. The PSII-3 sample was then incubated. *No* exogenous, chemical reductant (DTT) was added (method 1, R-NO). In Fig. 3, *A–C, lane 2,* urea and SDS were added to the methylamine-containing PSII-3 sample, the sample was incubated, and the reductant, DTT, was added *second* (method 2, R-2nd). In Fig. 3, *A–C, lane 3,* DTT was added *first*, the methylamine-containing PSII-3 sample was incubated, and urea and SDS were added (method 3, R-1st). In Fig. 3, *A–C, lane 4,* urea, SDS, and DTT were added *simultaneously* (method 4, SIM), and the PSII-3 sample was incubated. In Fig. 3, *A* and *C, lane 5,* as a negative control, a PSII-1 sample was treated by method 4 (SIM). AO and fatty acid-free BSA were also included in the SDS-PAGE experiment (Fig. 3, *A* and *C, lanes 6–9*). In *lanes 6* and *8,* AO and BSA were treated by method 2 (R-2nd); in *lanes 7* and *9,* AO and BSA were treated by method 4 (SIM). The total treatment time for each method was 90 min (Fig. 1).

As shown in a Coomassie-stained gel (Fig. 3*A*), this difference in the order of addition had no effect on the electrophoretic migration of AO and BSA (*lanes 6–9*). Most major PSII subunits were identifiable by their R_F values and molecular mass (see annotation, Fig. 3*A*) and were unaffected as well (Fig. 3*A, lanes 1–5*). However, minor alterations in the R_F values of some subunits were observed, especially when the PSII sample was not treated with DTT or treated with DTT following denaturation (compare lanes 1 and 2 with lanes 3–5). Many of these changes were observed between 40 and 50 kDa, which is the expected, apparent molecular mass for the CP47 subunit.

Therefore, in order to identify the location of CP47 in Fig. 3*B, lanes 1* and *2,* Western analysis was performed. This assay employed a monoclonal antibody that had been raised against CP47. The results demonstrate, that despite minor alterations in the pattern of Coomassie labeling (Fig. 3*A, lanes 1* and *2*), the R_F of CP47 was not appreciably affected by the four different treatment methods (Fig. 3*B*).

In Fig. 3*C,* we present the results obtained when PSII-3 was treated with [^{14}C]methylamine through the use of the four methods shown in Fig. 1. These data are representative of multiple SDS-PAGE experiments performed. Numerical data averaged from these experiments are also summarized in Table I. Overall, the amount of ^{14}C labeling of PSII-3 was 8–240-fold the extent of ^{14}C retained by the negative control, BSA (Table I), depending on the conditions employed. There was no significant difference in ^{14}C labeling when method 1 (Fig. 3*C, lane 1,* R-NO) was compared with method 2 (*lane 2,* R-2nd) or when method 3 (*lane 3,* R-1st) was compared with method 4 (*lane 4,* SIM). Observed ^{14}C labeling, when no chemical reductant was added, suggests that there may be another "endogenous" reductant produced by PSII under illumination, which competes effectively with DTT; this point is discussed in more detail below.

Treatment by method 3 (Fig. 3*C, lane 3*) and method 4 (*lane 4*) caused an approximately 8–11-fold decrease (Table I) in the amount of ^{14}C incorporated into CP47, when compared with methods 1 and 2. A 12–18-fold decrease in ^{14}C incorporation into D2/D1 subunits was also observed (Table I). This result suggests that DTT may slowly reduce a potential amine or aldehyde binding site and that this reduction may prevent binding of methylamine.

In the 30-min, method 4 treatment, the extent of ^{14}C incorporated into PSII-3 (Fig. 3*C, lane 4, SIM*), was increased 6–8-fold when compared with ^{14}C labeling of the negative control, PSII-1 (*lane 5, SIM*). This result was in qualitative agreement with the results of the experiment employing a 4-h incubation time with methylamine (Fig. 2), in which a 12-fold increase over PSII-1 was observed. Finally (Table I), the amount of ^{14}C incorporated into PSII-3 when method 3 (Fig. 3*C, lane 3, R-1st*) or method 4 (*lane 4, SIM*) was employed was comparable to the amount of ^{14}C incorporated into the positive control, AO (*lanes 6* and *7*). Although the order of addition had a dramatic effect on ^{14}C labeling of PSII-3, no significant effect was observed on ^{14}C incorporation into AO (Fig. 3*C, lanes 6* and *7*) or BSA (*lanes 8* and *9*). Therefore, data from the BSA lanes were averaged in numerical analyses.

Control experiments were performed to vary the time of incubation with methylamine before addition of denaturants (urea and SDS). No significant change in ^{14}C labeling of PSII-3 subunits was observed when the incubation time was varied between 60 and 15 min (data not shown). PSII-2 treated by method 4 has been shown previously to give a different result (13).

Purified plastoquinone migrates at the dye front in this gel system (13). Increased ^{14}C labeling near the dye front in Fig. 3*C, lanes 1* and *2,* may result from the interaction of plastoquinone and methylamine. Comparison of Fig. 3*C, lanes 1* and *2,* with *lanes 3* and *4* may imply that DTT slowly reduces plastoquinone and that plastoquinone reduction prevents methylamine binding.

To assess whether an observed, slight increased retention of pigments in Fig. 3*C, lanes 1* and *2,* influenced the number of ^{14}C counts retained, blots were washed with methanol. Methanol washing removed retained chlorophyll or carotenoid but did not reproducibly decrease the number of counts retained by PSII subunits (data not shown).

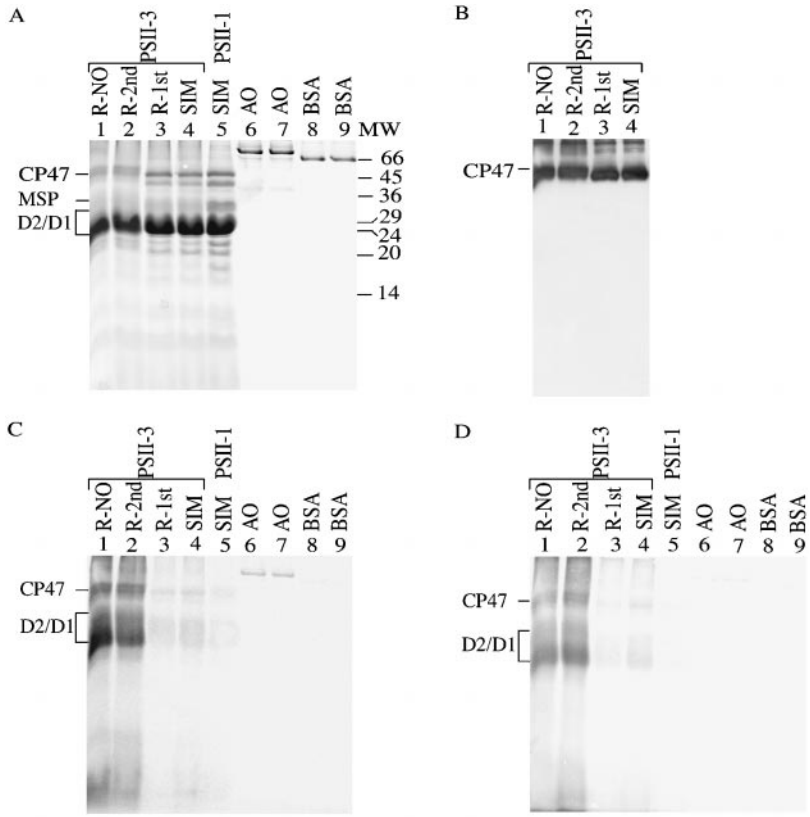

FIG. 3. **Methylamine and *t*-butylamine binding to PSII-3 and AO subunits.** Samples were incubated with 4 mM [^{14}C]methylamine (*A–C*) for 30 min or with 7.0 mM *t*-[^{14}C]butylamine (*D*) for 4 h. Samples were treated by a method described in Fig. 1, subjected to SDS-PAGE, and blotted onto Immobilon membranes. In *A,* the gel was stained with Coomassie Brilliant Blue. In *B,* a Western assay, employing a CP47 monoclonal antibody, is presented. In *C,* a phosphorimage shows ^{14}C labeling from methylamine. The data in *A–C* are derived from the same experiment. In *D,* a phosphorimage shows ^{14}C labeling from *t*-butylamine. In all panels: *lane 1,* PSII-3 (method 1); *lane 2,* PSII-3 (method 2); *lane 3,* PSII-3 (method 3); *lane 4,* PSII-3 (method 4); *lane 5,* PSII-1 (method 4); *lane 6,* AO (method 2); *lane 7,* AO (method 4); *lane 8,* BSA (method 2); *lane 9,* BSA (method 4). The electrophoretic migration of molecular mass standards (kDa) is indicated (*MW*).

TABLE I
^{14}C labeling of PSII subunits and AO

40 pmol of PSII-3 and 20 pmol of AO (40 pmol of AO monomer) were incubated in the presence of the appropriate amine as described in Fig. 1. Counts/pixel were obtained from quantitation of ^{14}C using a phosphorimager. These data were corrected for protein-free background and were normalized to any counts/pixel retained by a negative control (fatty acid-free BSA), which was run on the same gel. These data were averaged over multiple experiments; table entries are mean ± S.E. The number of measurements for *t*-butylamine, methylamine, and phenylhydrazine was 2, 4, and 4, respectively. The specific activities of [^{14}C] methylamine, *t*-butylamine, and phenylhydrazine were 57, 55, and 2.65 mCi/mmol, respectively. Detection limits were determined by ratioing BSA lanes, treated either by method 2 or 4, to each other for all the experiments performed (see under "Experimental Procedures" and Fig. 1); the limits were 1.6 ± 0.4 for methylamine, 1.1 ± 0.6 for *t*-butylamine, and 2.6 ± 2.3 for phenylhydrazine.

Subunits	Methylamine	*t*-Butylamine	Phenylhydrazine
CP47			
R-NO	90.4 ± 27.1	10.7 ± 1.8	6.0 ± 4.2
R-2nd	87.9 ± 15.8	15.8 ± 7.9	ND[a]
R-1st	8.4 ± 3.5	1.3 ± 0.1	ND
SIM	10.5 ± 7.7	1.9 ± 0.4	ND
D2/D1			
R-NO	236.2 ± 57.3	22.7 ± 6.0	10.5 ± 6.3
R-2nd	161.2 ± 39.2	24.8 ± 9.1	ND
R-1st	13.3 ± 8.7	2.0 ± 0.1	ND
SIM	13.1 ± 9.3	2.9 ± 0.2	ND
AO			
R-2nd	6.7 ± 1.4	0.5[b]	ND
SIM	6.3 ± 1.9	0.6[b]	10.5 ± 5.9

[a] ND, not determined.
[b] Value (mean) is below the detection limit.

Methylamine Binding Occurs During and After Protein Denaturation—To obtain more information about the conditions under which methylamine binding to PSII occurs, experiments were performed in which the amount of ^{14}C binding was measured when methylamine was added before (R-NO), during (DEN+MA), and after (DEN//MA) protein denaturation (Table II). Experiments were conducted under room lights (Table II, light). In the first experiment, methylamine was added 30 min before denaturation, denaturant (urea-SDS) was added, and the mixture was incubated for an additional 30 min (Table II, R-NO, light). This is the same treatment as employed in method 1 (Table I, R-NO) except that the incubation time in the presence of denaturant was a total of 30 min instead of 1 h. Compared with the longer incubation times, this 30-min incubation decreased ^{14}C labeling by a factor of approximately 1.8 (compare Table I, R-NO, and Table II, R-NO, light). This difference is attributable to the difference in denaturation time. In control experiments, the amount of ^{14}C labeling was shown

TABLE II
^{14}C labeling of PSII subunits from methylamine

40 pmol of PSII-3 and 20 pmol of AO (40 pmol of AO monomer) were incubated in the presence of methylamine. Counts/pixel were obtained from quantitation of ^{14}C using a phosphorimager. R-NO and SIM methods were the same as described in Fig. 1, except that the denaturing time was 30 min, and the total incubation time was 60 min instead of 90 min. In the method DEN+MA, methylamine was added during the denaturation of PSII-3, with no prior incubation. In the method DEN//MA, methylamine was added after the denaturation of PSII-3 but was not present before. The total time of methylamine incubation was 30 min in DEN+MA and DEN//MA. The numbers of measurements for R-NO, SIM, DEN+MA, and DEN//MA were 4–6, 4–6, 3, and 6, respectively.

Subunits	Light	Dark	Light/dark
CP47			
R-NO	50.0 ± 23.0	2.5 ± 0.4	ND[a]
SIM	6.6 ± 3.0	1.1[b]	ND
DEN + MA	14.5 ± 4.0	ND	ND
DEN//MA	16.3 ± 9.5	1.0[b]	1.7 ± 0.3
D2/D1			
R-NO	132.2 ± 42.7	9.5 ± 2.5	ND
SIM	6.7 ± 3.2	1.3[b]	ND
DEN + MA	36.5 ± 3.4	ND	ND
DEN//MA	39.4 ± 14.9	3.7 ± 1.6	4.7 ± 1.8

[a] ND, not determined.
[b] Value (mean) is below the detection limit, which is 1.6 ± 0.4 (see legend to Table 1).

to be linearly related to the time of incubation in the presence of denaturant, in the time range of 15–60 min (data not shown).

The results described above suggest that methylamine binding to PSII-3 can occur during protein denaturation. To test this idea, methylamine was added during a 30-min denaturation (Table II, DEN+MA, light) or after a 30-min denaturation (Table II, DEN//MA, light). The total incubation time with methylamine was the same (30 min) in both these experiments. Table II shows that the amounts of ^{14}C binding obtained with method DEN+MA and method DEN//MA were similar and were 14–39-fold the amount of ^{14}C retained by the negative control, fatty acid-free BSA. The amounts of ^{14}C binding obtained with method DEN+MA and method DEN//MA were approximately 3-fold less than the amount of ^{14}C binding obtained when methylamine was added before denaturation (Table II, R-NO). These results suggest that a significant amount of methylamine binding occurs during protein denaturation and that similar amounts of binding occur during and after protein denaturation.

The Light Reactions of PSII Alter Labeling from Methylamine—In our earlier work, retention of ^{14}C by PSII subunits was attributed to reductive trapping of amine substrate and/or aldehyde product by DTT (13). However, Fig. 3*C* shows that DTT addition is not necessary to retain ^{14}C, derived from methylamine, on CP47 and D2/D1 subunits (compare Fig. 3*C, lanes 1* and *2*). Assuming that Schiff bases are unstable during SDS-PAGE, one possible explanation for these results is that the PSII light reactions are a source of reducing equivalents for amine/aldehyde trapping. This is thermodynamically feasible, given the similar midpoint potentials of plastoquinone, an electron acceptor in PSII (28), and quinocofactors and their analogs (29–31).

Supporting these explanations, Table II shows that illumination increased the amount of ^{14}C retained by PSII subunits. When method 1 was employed (R-NO) in the *dark*, a 14–20-fold *decrease* in ^{14}C labeling of CP47 and D2/D1 subunits was observed, compared with ^{14}C incorporation in the light (Table II). When method 4 was employed (SIM), a dramatic reduction in ^{14}C labeling of CP47 and D2/D1 subunits in the dark was also observed. The dark incubation time, prior to methylamine addition, was 2.5 h. This is a long incubation time compared with the life-time of oxidized and reduced species in PSII-3 (32, 33).

Although light intensities were low in these experiments (4 $\mu E/m^2$-s), irreversible, oxygen-mediated modification of PSII was a possible explanation for the increase in ^{14}C incorporation observed under illumination. Such an effect would not reverse upon transfer of the sample to complete darkness for 30 min. On the other hand, if the light-induced effects were mediated by electron transfer reactions and/or by a conformational change (see below), then these light-induced effects would be reversible in this time period.

To investigate this point, illumination conditions were varied. In these experiments, methylamine was added after denaturation (DEN//MA). Table II presents the results of treatments, which were carried out in the light (30 min, DEN//MA, light), in the dark (30 min, DEN//MA, dark), or in a light alternation protocol in which the sample was first illuminated (30 min) and then dark-adapted (30 min, DEN//MA, light/dark). Methylamine was added either before the second dark adaptation (data not shown) or after the second dark adaptation (Table II). As shown, illumination of PSII-3 resulted in an approximately 13-fold increase in ^{14}C retention by CP47 and D2/D1. However, the amount of ^{14}C retained in samples that were illuminated and then were incubated in the dark was indistinguishable from the amount retained by samples that were not illuminated (Table II, DEN//MA, light/dark and dark). Similar results were obtained in a light alternation protocol in which methylamine was added before the second dark adaptation (data not shown). These results are consistent with reversible light-induced PSII changes in redox state leading to ^{14}C trapping. Another possible explanation is that the light reactions modulate access to the amine binding site through a protein conformation change. These two explanations are not mutually exclusive.

In method 4 (SIM), DTT was added to samples at the same time as protein denaturation was initiated. The experiments in Table II show that illumination effects were observed under method 4 conditions, with a significant stimulation of ^{14}C retention in the light compared with dark-adapted samples. This result implies that DTT is inefficient as a reductant for the ^{14}C adduct compared with light induced reductants and/or that light-induced conformational changes modulate binding.

The Order of Addition Affects ^{14}C Labeling from t-Butylamine—PSII-3 and PSII-2 exhibit amine oxidation activity, and the aldehyde product was isolated and quantitated (13). Therefore, there are three possible explanations for the stimulation of ^{14}C labeling when denaturation was carried out in the absence of exogenous reductant (method 1) or before the addition of exogenous reductant (method 2). The first explanation is that the PSII-3 labeling, observed after incubation with [^{14}C]methylamine, was derived from the production and the binding of [^{14}C]aldehyde. The second explanation is that the stimulation of ^{14}C incorporation under these conditions was due to increased [^{14}C]methylamine binding. The third possibility is that binding of both [^{14}C]amine and [^{14}C]aldehyde was involved in this phenomenon (13).

To distinguish among these alternatives, *t*-[^{14}C]butylamine was employed. This compound cannot be oxidized to produce an aldehyde via a transamination mechanism because it lacks an abstractable proton at the Cα position (29, 34–38). This amine has been shown to covalently bind to a quinone model compound, forming a Schiff base (39).

The results of treatment with *t*-butylamine are presented in Fig. 3*D* and Table I. Treatment of PSII-3 with *t*-[^{14}C]butylamine resulted in the covalent incorporation of ^{14}C into PSII subunits, D2/D1 and CP47. Overall, the amount of labeling was 2–25-fold the extent of ^{14}C retained by the negative control, BSA (Table I), depending on the conditions employed. Note that the amount of ^{14}C incorporation from *t*-butylamine into PSII subunits was decreased approximately 4–10-fold compared with ^{14}C incorporation from methylamine under the same conditions (Table I). As observed with [^{14}C]methylamine (Fig. 3*C, lanes 1* and *2*), there was no significant difference in ^{14}C labeling when method 1 (Fig. 3*D, lane 1, R-NO*) was compared with method 2 (Fig. 3*D, lane 2, R-2nd*). There was a small but statistically significant decrease in ^{14}C labeling when method 3 (Fig. 3*D, lane 3, R-1st*) was compared with method 4 (*lane 4, SIM*), but the amount of CP47 labeling observed in method 3 was close to the detection limit (Table I). Treatment by method 1 (Fig. 3*D, lane 1, R-NO*) and method 2 (*lane 2, R-2nd*) caused a 6–12-fold increase in the amount of ^{14}C incorporated into CP47, compared with methods 3 and 4 (*lanes 3* and *4*, Table I). An 8–12-fold stimulation of ^{14}C incorporation into D2/D1 was also observed (Table I). The amount of ^{14}C incorporation into PSII-3, when denaturation and reduction were performed simultaneously (Fig. 3*D, lane 4, SIM*), was increased 2-fold when compared with the amount of ^{14}C incorporated into the negative control, PSII-1 (*lane 5*). Finally, there was no significant incorporation of ^{14}C into AO (Fig. 3*D, lanes 6* and *7*), and the order of addition did not alter this result (Table I). This result

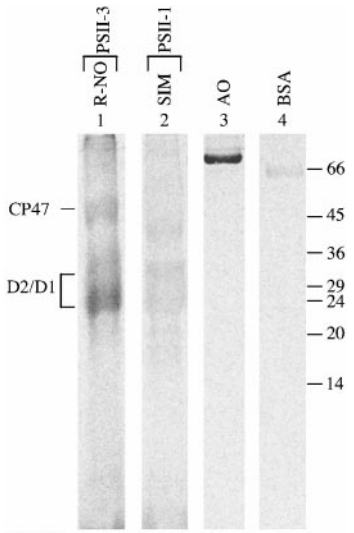

FIG. 4. **Phenylhydrazine binding to PSII-3 and AO subunits.** Samples were incubated with 56.8 μM [^{14}C]phenylhydrazine for 30 min, subjected to SDS-PAGE, and blotted to Immobilon membranes. Methods 1 and 4 (Fig. 1) were employed. The phosphorimage is shown. *Lane 1,* PSII-3 (method 1); *lane 2,* PSII-1 (method 4); *lane 3,* AO (method 4); *lane 5,* BSA (method 4). The electrophoretic migration of molecular mass standards (kDa) is indicated at the *right*.

is attributed to the bulky nature of *t*-butylamine (39), which may not have sufficient access to the topaquinone cofactor in either the folded or in the denatured state (for structures of some amine oxidases, see Refs. 40–42).

Phenylhydrazine Labeling of PSII Subunits—Phenylhydrazine is a reagent that will modify amino acid side chains containing activated carbonyl groups. The specific activity of commercially available [^{14}C]phenylhydrazine was a factor of 21 lower than the specific activity of *t*-[^{14}C]butylamine and methylamine. This low specific activity limited our ability to detect and interpret small differences in phenylhydrazine binding. Therefore, only method 1 was employed in the phenylhydrazine experiments (Fig. 4). Fig. 4 and Table I show that treatment of PSII-3 with [^{14}C]phenylhydrazine resulted in the incorporation of a significant amount of ^{14}C into the PSII subunits, D2/D1 and CP47. The amount of CP47 labeling was 6-fold the amount of ^{14}C retained by the negative control, BSA; the amount of D2/D1 labeling was 10-fold the amount of ^{14}C retained by BSA (Fig. 4, *lanes 1* and *4*; Table I). The amount of ^{14}C incorporation into PSII-3 was approximately 3-fold the amount of ^{14}C retained by the negative control, PSII-1 (Fig. 4, *lanes 1* and *2*; Table I). Significantly, the extent of PSII-3 labeling from phenylhydrazine was similar to the extent of AO labeling (Fig. 4, *lanes 1* and *3*; Table I).

Phenylhydrazine was added to PSII-3 before and 30 min after denaturation. This experiment was carried out under room illumination. An apparent 2-fold increase in the level of ^{14}C labeling was observed when phenylhydrazine was added to PSII-3 after denaturation (data not shown). Because denaturation inactivates the PSII light reactions, this result indicates that ^{14}C labeling was not caused by a light reaction-mediated, one-electron oxidation of the phenylhydrazine ring. When reactions were carried out in strict darkness, only an apparent 3-fold decrease in ^{14}C labeling was observed (data not shown). This decrease is much less significant than the 13-fold decrease observed with methylamine. These data are consistent with the formation of a stable phenylhydrazone in PSII.

Comparative Quantitation of Amine and Phenylhydrazine Binding—Comparison to a ^{14}C BSA standard curve (13) was performed in order to estimate the number of binding sites for

TABLE III
Quantitation of methylamine, t-butylamine, and phenylhydrazine binding

Samples containing 40 pmol of PSII-3 were incubated for 4 h with t-butylamine and for 30 min with methylamine or phenylhydrazine (method 1, R-NO, Fig. 1). The numbers of measurements for *t*-butylamine, methylamine and phenylhydrazine were 2, 4, and 4, respectively. The values given are in units of percent (mol/mol × 100) ± S.E.

Subunit	Methylamine	*t*-Butylamine	Phenylhydrazine
CP47			
R-NO	58 ± 17	7.3 ± 1.2	84 ± 58
D2/D1			
R-NO	153 ± 37	15.2 ± 3.9	147 ± 88

t-butylamine, methylamine, and phenylhydrazine on PSII subunits under denaturing conditions. In these calculations, a one-to-one stoichiometry of CP47, D2, and D1 per PSII reaction center was assumed (43). The results are shown in Table III. Under denaturing conditions, 58–84% of CP47 and 147–153% of D2/D1 were labeled by [^{14}C]methylamine and [^{14}C]phenylhydrazine (Table III). By contrast, 7% of CP47 and 15% of D2/D1 were labeled with *t*-[^{14}C]butylamine (Table III).

DISCUSSION

Our previous work employed methylamine and benzylamine and demonstrated that these amines interact with PSII subunits to give covalent adducts (13). Direct evidence for amine oxidation was also obtained through isolation of the ^{14}C-labeled benzaldehyde product of [^{14}C]benzylamine oxidation. Electron paramagnetic resonance control experiments provided no evidence that these reactions were occurring through high potential unmodified tyrosyl radicals (Z and D) in PSII. Therefore, these reactions were ascribed to the presence of an activated carbonyl group in one or more PSII subunits. We suggested that access to these sites was controlled by the 18- and 24-kDa polypeptides and by chloride. Removal of MSP and manganese increased amine interaction with these putative carbonyl groups, which were attributed to posttranslational modification of one or more amino acid residues at the active site (13).

Model compound studies show that amines can interact with quinones in a variety of ways. Amines can be one-electron-oxidized by high potential quinones (see Refs. 35 and 36 and references therein). These reactions can proceed through direct electron transfer reactions and can lead to the production of paramagnetic species (35). However, the midpoint potentials for primary amines are in the 1.2–2.0 V *versus* Ag/0.1 Ag^+ range (36), making it unlikely that any PSII component would be competent in such a reaction. Our previous electron paramagnetic resonance control experiments also argue against such a mechanism (13). Amine oxidation reactions can also proceed via hydrogen atom and hydride transfer reactions, but these reactions also require high potential oxidants (35, 36).

p-Quinones can undergo amine nucleophilic addition reactions directly to the quinoid ring (35). *o*-Quinones and substituted *p*-quinones can undergo amine adduct formation to give a carbinolamine derivative (15, 29, 30, 35, 36, 38, 39). This derivative can then form an iminoquinone intermediate or undergo a direct α-proton abstraction in the addition/elimination pathway (29, 34–38, 44). The iminoquinone intermediate can rearrange to give an imine, which can be converted to aldehyde and aminophenol products by hydrolysis. Other reaction pathways also exist; these involve a second addition of an amine to the iminoquinone and the imine intermediates. The tendency to undergo these different reactions is influenced by the properties of the amines, including the acidity of the α-proton and steric bulk. Hydrazines are particularly prone to the formation of stable adducts (29, 34–38, 44).

Amine oxidation by a transamination pathway, which gives

aldehyde and aminophenol products, or by an addition/elimination reaction, which gives an oxidized amine and a quinol, requires the abstraction of a proton on the amine α carbon. Because *t*-butylamine lacks this proton, it would not be expected to be reactive in the transamination or the addition/elimination pathway. However, *t*-butylamine has been shown to react *in vitro* with a methoxy-substituted *o*-quinone (39) and other *o*-quinones (38) to form a Schiff base complex. Substituted *p*-quinones formed only amine salts with *t*-butylamine (39). This difference was attributed to the steric bulk of *t*-butylamine, which appeared to decrease its reactivity. Note that amine salts are not expected to survive the conditions of denaturing SDS-PAGE, but only covalent adducts are expected to be detectable.

In this study, we have used three different amines, methylamine, *t*-butylamine, and phenylhydrazine, to obtain chemical information about the primary structure of PSII subunits. Methylamine can be oxidized to form formaldehyde. Therefore, ^{14}C labeling from [^{14}C]methylamine could be due to either amine or formaldehyde labeling under reductive conditions. However, phenylhydrazine and *t*-butylamine cannot be oxidized by that same mechanism. In comparison to our earlier work (13), we have employed a more sensitive SDS-PAGE assay method. This method allows the interaction of phenylhydrazine and bulky amines with PSII to be monitored. This is especially important in the case of phenylhydrazine, because the commercially available ^{14}C labeled reagent is of low specific activity.

The work presented here shows that ^{14}C derived from [^{14}C]methylamine, *t*-butylamine, and phenylhydrazine is covalently incorporated into the PSII subunits CP47 and D2/D1. Because both phenylhydrazine and *t*-butylamine tag CP47 and D2/D1, our results imply that [^{14}C]amine-derived adducts are formed in these subunits. Low intensity room illumination dramatically increased ^{14}C labeling of PSII from methylamine, and this effect was found to be reversible. Only a small effect was observed on ^{14}C labeling from phenylhydrazine, however. These illumination-induced increases suggest that PSII light reactions alter the conformational and redox state of the enzyme and facilitate trapping of the amine adduct and binding of the phenylhydrazine.

It is particularly interesting that phenylhydrazine binds to PSII subunits. Our data are consistent with the formation of a stable phenylhydrazone, because predenaturation and inactivation of PSII actually slightly increases the extent of ^{14}C phenylhydrazine binding. This experiment argues against the interpretation that ^{14}C labeling is caused by the light-driven one-electron oxidation of the phenylhydrazine aromatic ring by PSII. ^{14}C labeling caused by formation of a phenylhydrazone has been taken as diagnostic of the presence of a carbonyl-containing moiety (for example, see Ref. 45). Formation of the phenylhydrazone during phenylhydrazine titration of AO has been monitored by an increase in absorbance at 450 nm (46). However, monitoring of phenylhydrazine binding to PSII by this approach is not possible due to spectral interference from pigments associated with PSII (47, 48).

Our quantitative results suggest that the interaction of methylamine and phenylhydrazine with PSII subunits approaches a one-to-one stoichiometry under denaturing conditions and that PSII contains at least three modified amino acids, which can react with amines and phenylhydrazine. Although the gel system employed does not separate D1 and D2, the amino acid sequences are similar, and D1 and D2 form a symmetrical heterodimeric core for PSII (49). Thus, it is reasonable to propose one binding site on each subunit with fractional occupation of each, although these results do not rule out other possibilities.

^{14}C incorporation into PSII-3 from methylamine and *t*-butylamine dramatically increased when PSII-3 was denatured before the addition of an exogenous electron donor. The increased level of ^{14}C labeling may be a result of greater access to the binding site upon denaturation of PSII. However, the level of ^{14}C incorporation from *t*-butylamine is less than the level of ^{14}C binding from methylamine. This work suggests that steric hindrance may still limit binding of this bulky amine to denatured PSII-3. Taken together, the phenylhydrazine and *t*-butylamine data provide support for the proposal that PSII contains quinocofactors and that amines and hydrazine reagents have limited access to these sites in the functional enzyme.

The presence of modified amino acids at the PSII active site (13) may have important implications concerning the mechanism of water oxidation. Such cofactors could bind metal atoms, play roles in electron transfer reactions, or directly participate in water oxidation reactions. In future work, the structure and location of the posttranslationally modified amino acid residues will be determined.

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