

Location of chloride and its possible functions in oxygen-evolving photosystem II revealed by X-ray crystallography

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The chloride ion, Cl⁻, is an essential cofactor for oxygen evolution of photosystem II (PSII) and is closely associated with the Mn₄Ca cluster. Its detailed location and function have not been identified, however. We substituted Cl⁻ with a bromide ion (Br⁻) or an iodide ion (I⁻) in PSII and analyzed the crystal structures of PSII with Br⁻ and I⁻ substitutions. Substitution of Cl⁻ with Br⁻ did not inhibit oxygen evolution, whereas substitution of Cl⁻ with I⁻ completely inhibited oxygen evolution, indicating the efficient replacement of Cl⁻ by I⁻. PSII with Br⁻ and I⁻ substitutions were crystallized, and their structures were analyzed. The results showed that there are 2 anion-binding sites in each PSII monomer; they are located on 2 sides of the Mn₄Ca cluster at equal distances from the metal cluster. Anion-binding site 1 is close to the main chain of D1-Glu-333, and site 2 is close to the main chain of CP43-Glu-354; these 2 residues are coordinated directly with the Mn₄Ca cluster. In addition, site 1 is located in the entrance of a proton exit channel. These results indicate that these 2 Cl⁻ anions are required to maintain the coordination structure of the Mn₄Ca cluster as well as the proposed proton channel, thereby keeping the oxygen-evolving complex fully active.

membrane proteins | oxygen evolution | photosynthesis | manganese enzyme

Photosynthetic water oxidation produces the oxygen required for life on the earth and is catalyzed by Photosystem II (PSII), a multiprotein complex with a total molecular mass of 350 kDa (1). The catalytic center for water splitting, i.e., the oxygen-evolving complex (OEC), is located in the membrane surface of the luminal side of thylakoid membranes and is composed of 4 Mn atoms and 1 Ca atom in the protein matrix of PSII. Oxygen is produced from 2 molecules of water by sequential, light-induced electron-abstracting events at the Mn₄Ca cluster that cycle through the S_i states with i = 0–4 (2). One or more Cl⁻ ions are required for the water oxidation cycle to proceed, a requirement not often found in other biological systems (3, 4). Depletion of Cl⁻ has been shown to inhibit the S₂ → S₃ and S₃ → S₀ transitions (5). The roles of Cl⁻ proposed for OEC include ligation to Mn or Ca atoms (6–8), regulation of the redox potential of the Mn₄Ca cluster (9), maintaining a hydrogen bond network (10), and activation of the substrate water (11). In addition, an association of Cl⁻ with amino acid residues in the Mn coordination shell has been proposed (12, 13).

The structure of PSII, including the Mn₄Ca cluster, has been analyzed by x-ray crystallography (14–17). These studies, together with polarized extended x-ray absorption fine structure (EXAFS) measurements (18), have provided much information about the structure of the Mn₄Ca cluster. However, they did not provide any information about the number and location of Cl⁻ ions within the OEC. The inhibition of particular S-state transitions upon depletion of Cl⁻ has been taken as evidence of the close proximity of Cl⁻ to the Mn₄Ca cluster, a proximity that also was suggested by studies of electron spin echo envelope modulation (19, 20) and Fourier transform infrared spectroscopic

studies (21). Recent EXAFS studies, however, suggested that Cl⁻ ions do not bind to the Mn₄Ca cluster in its S₁ state, and that no Cl⁻ is present within a distance of 5 Å from the metal center (13). Thus, the exact location of Cl⁻ ions in PSII remains to be resolved.

The stoichiometry of Cl⁻ associated with PSII is unclear also because of the difficulty of directly detecting Cl⁻. Lindberg et al. have demonstrated that PSII membranes isolated from spinach grown on a medium containing ³⁶Cl⁻ retained about 1 Cl⁻/PSII (22). They also proposed that this Cl⁻ site can be either high affinity (K_d = 20 μM) and slowly exchanging (t_{1/2} = 1 h) or low affinity (K_d = 0.5 mM) and rapidly exchanging (t_{1/2} < 15 sec) (23). Even in the Cl⁻-depleted condition, however, the oxygen-evolving activity of PSII membranes reported in these studies remained at 35% of the control level. This result raises the possibility that the Cl⁻ ions associated with PSII were not completely removed from the samples under the conditions used.

The function of Cl⁻ in the OEC can be partially substituted by other anions, with the efficiency being Cl⁻ ≈ Br⁻ ≫ NO₃⁻ > NO₂⁻ > I⁻ (3, 24). This phenomenon can be used to study the Cl⁻ binding in the OEC, and indeed most previous studies have substituted Br⁻ or other anions for Cl⁻ to investigate its binding and function in the OEC. The substitution of Cl⁻ by Br⁻ and I⁻ is particularly useful in studying the coordination structure of Cl⁻ in OEC by x-ray crystallography, because Br⁻ has a K-edge absorption wavelength of 0.92 Å, and I⁻ has significant effects of anomalous dispersion around this wavelength that fall into the x-ray wavelength routinely used for structural studies. Indeed, in a recent study using x-ray crystallography, Murray et al. (25) substituted Br⁻ for Cl⁻ in PSII and identified 2 anion-binding sites in the vicinity of the OEC. In the present study, we substituted Br⁻ or I⁻ for Cl⁻, using purified PSII dimer from a thermophilic cyanobacterium, *Thermosynechococcus vulcanus*, and analyzed the crystal structure of the substituted PSII at higher resolutions. Both the difference Fourier maps between native PSII (Cl⁻-PSII) and PSII crystals with Br⁻ or I⁻ substitutions and the anomalous Fourier maps of PSII with Br⁻ and I⁻ substitutions revealed the presence of 2 anion-binding sites in the vicinity of the Mn₄Ca cluster. Because oxygen evolution was inhibited completely by substitution of Cl⁻ with I⁻ and was recovered on re-substitution of I⁻ by either Cl⁻ or Br⁻, our

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Data deposition: The structures of Br-substituted and I-substituted PSII have been deposited with the PDB under accession numbers of 3A0B and 3A0H, respectively.

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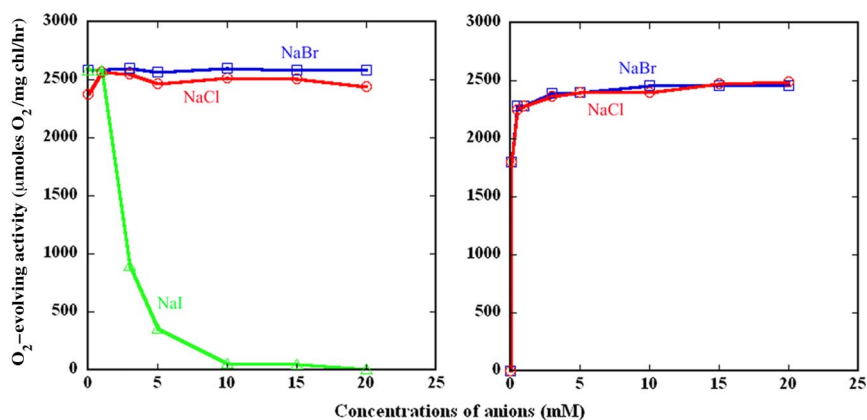


Fig. 1. Effects of Br^- substitution or I^- substitution and Cl^- or Br^- reconstitution on oxygen evolution of a PSII dimer from *T. vulcanus*. (Left) Effects of Br^- or I^- substitution on oxygen evolution measured in the presence of respective anions. O, Cl^- -containing PSII; \square , PSII with Br^- substitution; Δ , PSII with I^- substitution. (Right) Recovery of oxygen evolution of PSII with I^- substitution by Cl^- (O) and Br^- (\square).

results indicate that these 2 anion-binding sites represent the Cl^- -binding sites in native PSII.

Results

Substitution of Cl^- by Br^- and I^- . Cl^- in the PSII core dimer was replaced by Br^- or I^- by a washing procedure with solutions containing either Br^- or I^- instead of Cl^- (See *Materials and Methods*.) The resulting samples were analyzed for their oxygen-evolving activities in the presence of either Br^- for the Br^- -substituted PSII or I^- for the I^- -substituted PSII. Fig. 1 shows that, although PSII showed 90% of the activity when measured in a Cl^- -free medium because of Cl^- contamination of the medium (which was estimated to be $\approx 30\text{--}50\ \mu\text{M}$), and Br^- substitution did not affect the oxygen-evolving activity significantly, I^- substitution affected the activity remarkably: the activity was similar to Cl^- -PSII at I^- concentrations lower than 1 mM but decreased rapidly as the concentration of I^- in the measuring medium increased and was inhibited completely in the presence of 15–20 mM I^- . This finding is in agreement with previous reports (12) and indicates that the Cl^- associated with PSII is replaced completely by I^- in the presence of 15–20 mM I^- . The activity of PSII with I^- substitutions was recovered by washing the I^- -substituted PSII with a I^- -free medium and supplementing the measuring medium with either Cl^- or Br^- . A recovery of 70% activity was achieved even in the presence of less than 0.1 mM Cl^- or Br^- in the measuring buffer, and a full recovery of activity was achieved by 1–3 mM of either of the 2

anions in the concentration. These results indicate that the association of I^- with PSII is reversible, and both Cl^- and Br^- can replace I^- to support PSII oxygen evolution with a high affinity. The recovery of activity by simply supplying Cl^- or Br^- in the measuring medium also indicates that no reduction or release of the Mn atoms occurs upon I^- substitution and that I^- is inhibited by its reversible binding to the Cl^- -binding site. Cl^- and Br^- were equally effective in recovering the oxygen-evolving activity, in agreement with the previous and present finding that Br^- can support oxygen evolution as effectively as Cl^- . This finding indicates that Cl^- , Br^- , and I^- bind to the same functional site in PSII.

Structural Analysis of PSII with Br^- and I^- Substitutions. PSII with Br^- or I^- substitutions was crystallized in the presence of a total concentration of 40 mM of each anion (20 mM NaBr or NaI plus 10 mM CaBr_2 or CaI_2 , see *Materials and Methods*), ensuring a complete substitution of Cl^- by Br^- or I^- . The crystallization conditions for PSII with Br^- or I^- substitutions were similar to those for native PSII (Cl^- -PSII) (15, 26), and the resulting PSII crystals with Br^- and I^- substitutions belonged to the same space group and had similar unit cell constants as the native PSII (Cl^- -PSII) crystals (Table 1). Diffraction data from PSII with Br^- substitutions had a resolution of 3.7 Å, and diffraction data from PSII with I^- substitutions had a resolution of 4.0 Å (Table 1). Using the phase information calculated from the PSII structure without Br^- or I^- , we were able to calculate the

Table 1. X-ray crystallographic data of PSII crystals from wild-type and Br- and I-PSII

Data collection	Native PSII-1 ^a	Br-PSII	Native PSII-2 ^a	I-PSII
X-ray source	BL41XU	BL41XU	BL41XU	BL41XU
Wavelength	0.9 Å	0.9 Å	1.0 Å	1.0 Å
Resolution (Å) ^b	50–4.0	50–3.7	50–4.0	50–4.0
Unique reflections	73143	87773	68269	74602
Rmerge (%) ^b	8.2 (71.0)	7.2 (69.6)	8.2 (54.2)	9.1 (71.5)
$I/\sigma(I)$ ^a	21.7 (1.8)	28.8 (2.2)	22.8 (2.0)	23.1 (2.2)
Completeness ^b	94.7 (86.2)	91.7 (82.6)	91.7 (77.4)	99.0 (99.3)
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)				
<i>a</i>	130.0	129.6	128.6	128.5
<i>b</i>	225.6	224.6	225.7	224.7
<i>c</i>	307.2	302.9	304.5	304.9

^aNative PSII-1 is the crystal used to calculate the difference Fourier map between Cl^- PSII and Br^- -substituted PSII. Native PSII-2 is the crystal used to calculate the difference Fourier map between Cl^- PSII and I^- -substituted PSII.

^bNumbers in parenthesis represent the highest-resolution shell.

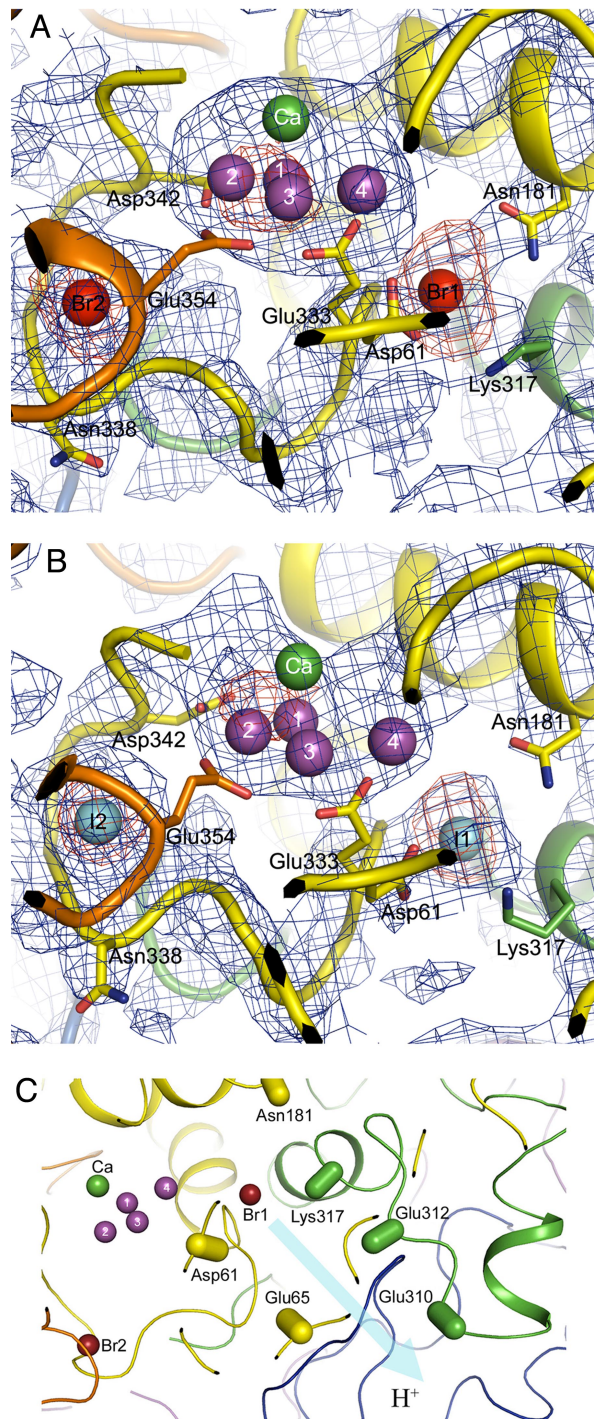


Fig. 3. Location of the 2 anion-binding sites in PSII. (A) Composite omit Fo-Fc map (blue) and anomalous map (red) of PSII with Br⁻ substitution contoured at $\sigma = 1.0$ and 4.0, respectively, superimposed on the structure of the Mn₄Ca cluster and its surrounding regions. The structure of PSII with Br⁻ substitution was shown by the molecular replacement method with the structure of the PDB code 2AXT as the search model. Color codes for the residues are as follows: yellow, D1; green, D2; orange, CP43; purple, Mn atoms. (B) Composite omit Fo-Fc map (blue) and anomalous map (red) of I⁻ substituted PSII contoured at $\sigma = 1.0$ and 4.0, respectively, superimposed on the structure of the Mn₄Ca cluster and its surrounding regions. The structure of PSII with I⁻ substitution was shown by the molecular replacement method. The color codes for the residues are the same as in A. (C) Location of Br₁ relative to the proton exit channel proposed in (16, 27–29). The residues of PsbO are drawn in blue, and the residues of other subunits are in the same colors as in A. The directions of atoms from C α to C β in the residues are indicated by capsule-shaped objects.

Table 3. Distances of the 2 anion-binding sites from the Mn₄Ca-cluster. The number of Mn atoms corresponds to those labeled in Fig. 3

	Distances (Å)
Mn ₄ -Br ₁	6.4
Mn ₄ -I ₁	7.4
Mn ₂ -Br ₂	7.3
Mn ₂ -I ₂	6.5
Br ₁ -Br ₂	14.6
I ₁ -I ₂	13.9
Ca-Br ₁	9.4
Ca-I ₁	10.1
Ca-Br ₂	11.1
Ca-I ₂	9.6

the main chain nitrogen of D1-Asn-338, suggesting that the Br₂ (and I₂) may be coordinated by these 2 residues. The side chain of CP43-Glu-354 is known to coordinate the Mn₄Ca cluster. The main chain nitrogen of D1-Asp-342 is 6.7 Å apart from Br₂ (I₂), suggesting that it may not be involved directly in the coordination of Br₂ (I₂) but may constitute the second coordination shell. The 2 Cl atoms are relatively far from their first coordination shell, suggesting that the 2 anions may be coordinated by the surrounding amino acid residues through aqua-ion or hydrogen bonding.

Additional sites found to bind I⁻ in I⁻ substituted PSII were identified as CP47-Cys-112, PsbTc-Cys-12, and D2-Tyr-160 (Y_D) (Fig. 2B and Fig. 4). The electron density of I⁻ associated with D2-Tyr-160 was weaker than at the other 2 sites. The distances between I⁻ and CP47-Cys-112 and between I⁻ and PsbTc-Cys-12 are rather short, suggesting a direct binding of I⁻ to the side chain of these 2 Cys residues. These sites may not be involved in oxygen evolution and thus may not be relevant to the functional Cl⁻-binding sites (see Discussion).

Discussion

We demonstrated here that there are 2 anion-binding sites on either side of the Mn₄Ca cluster at a similar distance from the nearest Mn atom and from the Ca atom. We conclude that these 2 sites bind the functional Cl⁻ ions required for oxygen evolution because substitution by I⁻ completely abolished oxygen evolution and the inhibited activity could be recovered simply by replacing I⁻ with Cl⁻ or Br⁻. These results indicate that in our PSII functional Cl⁻ was replaced effectively by Br⁻ and I⁻. The 2 sites revealed here are consistent with a very recent report showing 2 Br-binding sites around the Mn₄Ca cluster (25). Our structural analysis was performed at a higher resolution (i.e., 3.7 Å–4.0 Å vs. 4.21–4.45 Å). We also used I⁻ as a substitute for Cl⁻, and this substitution inhibited oxygen evolution completely. This finding allowed us to correlate these binding sites directly to oxygen evolution.

Because of possible radiation damage caused by the X-rays used, we cannot completely exclude the direct binding of Cl⁻ to the Mn₄Ca cluster. However, the 2 Cl⁻-binding sites identified here have significant functional consequences. The similar locations of these 2 Cl⁻-binding sites and their closeness to the Mn₄Ca cluster suggest that they both may be important for the normal function of the OEC. This importance is supported by the fact that the 2 anion-binding sites are surrounded by residues that either are coordinated directly with the Mn₄Ca cluster or are in close proximity to the cluster. Because 1 of the residues coordinated with Cl₂ is the main chain of CP43-Glu-354, and its side chain is known to coordinate the Mn₄Ca cluster, Cl⁻ may be required to maintain the structure needed for the correct coordination of this residue with the Mn₄Ca cluster. The removal

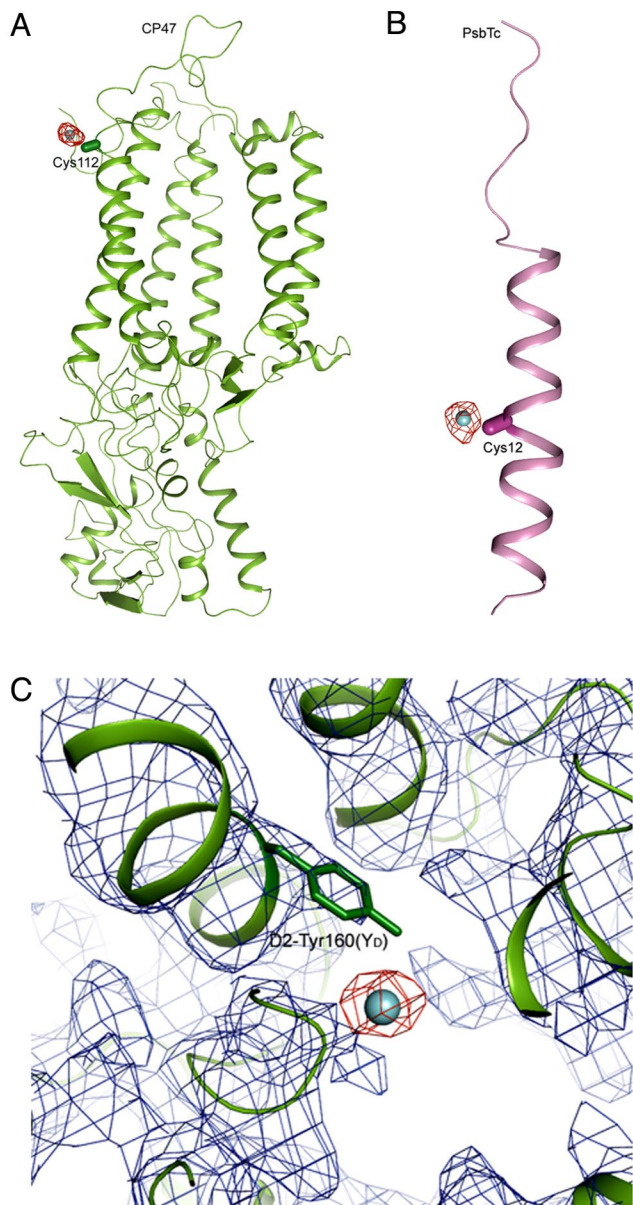


Fig. 4. Additional binding sites of I^- in I^- -substituted PSII. (A) Fo-Fc omit map of PSII with I^- substitution (red) contoured at $\sigma = 3.0$, superimposed on the structure of CP47 (green) obtained by the molecular replacement method, showing the association of I^- with CP47-Cys-112. (B) Fo-Fc omit map of PSII with I^- substitution (red) contoured at $\sigma = 3.0$, superimposed on the structure of PsbTc (pink), showing the association of I^- with PsbTc-Cys-12. The capsule-shaped objects show the direction of atoms from $C\alpha$ to $C\beta$ in the 2 Cys residues. (C) 2Fo-Fc map (blue, $\sigma = 1.0$) and omit Fo-Fc map (red, $\sigma = 2.0$) of PSII with I^- substitution around the region of D2-Tyr-160 (Y_D), together with the structure of D2 (green) in this region.

of Cl^- from this site or the replacement of Cl^- by I^- may affect the effective coordination of this residue to Mn. A similar situation is found in the Cl_1 -binding site, because 1 of the residues coordinated with this site is D1-Glu-333, whose side chain is coordinated with the Mn_4Ca cluster. Furthermore, recent studies have identified a possible proton exit channel from the Mn_4Ca cluster that involves D2-Lys-317, D1-Asp-61 as well as D1-Asn-181 (16, 27–29). Cl_1 is located in the entrance of the proton exit channel from the metal cluster (Fig. 3C). Cl or Br may be required to maintain the structure of this channel, and the binding of I^- instead of Cl^- or Br^- at this site may block the

proton exit pathway either because of a larger ion radius (2.20 Å for I^- vs. 1.81 Å for Cl^- or 1.96 Å for Br^-) or because of a protein conformational change induced by the binding of the heavier I^- anion. Alternatively, because I^- is heavier than Cl^- , binding of I^- at this site might lower the flexibility of the protein matrix. That flexibility might be required for conformational changes that are necessary for the functional S-state transitions to occur.

The distance of the 2 anion-binding sites from the Mn_4Ca cluster are consistent with a recent EXAFS study that found that Cl^- is not present within 5 Å of the Mn_4Ca cluster (13), suggesting that a direct interaction of the anion with the metal cluster is rather unlikely. Also, because these anions are rather far from the nearby residues, no covalent bond is likely to exist for their coordination. This evidence suggests that Cl^- is bound rather weakly to the PSII, which explains the easy release of Cl^- from binding sites in the PSII and the easy substitution of Cl^- by Br^- or I^- , and vice versa. This weak bonding may represent a normal feature of halide binding in proteins.

The 3 additional I^- -binding sites found here can be excluded for binding of functional Cl^- for several reasons. First, Br^- did not bind to these sites in Br^- -substituted PSII. Because Br^- can fully support oxygen evolution, this lack of binding indicates that functional Cl^- cannot be related with these residues. Second, all 3 sites are distant from the Mn_4Ca cluster, making a direct interaction of these residues with the OEC unlikely. Finally, oxygen evolution is still active even in the deletion mutant of cyanobacteria lacking the PsbTc subunit (30) and in site-directed mutants of Y_D in which binding of Cl^- would become impossible if these residues do coordinate Cl^- in native PSII. In fact, the association of I^- with Y_D is consistent with previous results that Y_D is iodinated in the dark (31). The weak electron density of I^- associated with Y_D may reflect the fact that the crystals were grown in the dark for several days, during which time part of Y_D^+ would have decayed to Y_D , resulting in a partial iodination of this residue. Iodination of another tyrosine residue, Y_Z , under illumination also was reported previously (31, 32). However, because the crystals were grown in the complete darkness, we did not observe electron density attributable to I^- at the Y_Z site.

The observation of the 3 additional I^- -binding sites not relevant to oxygen evolution suggests that the substitution of Cl^- by I^- in our experimental conditions was rather complete and reinforces our conclusion that the 2 anion-binding sites surrounding the Mn_4Ca cluster identified here represent the true Cl^- -binding sites in native PSII.

Materials and Methods

Purification and Crystallization of PSII. PSII dimer was purified from the thermophilic cyanobacterium *Thermosynechococcus vulcanus* according to methods described by Shen et al. (26, 33) and was suspended in a medium containing 20 mM MES (pH 6.0), 20 mM NaCl, and 3 mM $CaCl_2$ (medium A). For substitution of Cl^- by Br^- or I^- , the PSII samples were diluted to a concentration of 1 mg chlorophyll (chl)/mL with a medium containing 20 mM MES (pH 6.0), 20 mM NaBr, and 10 mM $CaBr_2$ (medium B, for Br substitution) or 20 mM MES (pH 6.0), 20 mM NaI, and 10 mM CaI_2 (medium C, for I substitution), mixed with the same volume of precipitation buffer (containing 26% PEG 1450 in medium B or C), and incubated for 30 min on ice in the dark. After incubation, PSII was precipitated by centrifugation at $15,000 \times g$ for 15 min, washed again with medium B or C, and finally suspended in either medium B or C at a concentration of 4.0 mg chl/mL.

Crystallization of PSII dimer was performed using the hanging drop vapor diffusion method. PSII samples were mixed with the same volume of crystallization buffer containing 20 mM MES (pH 6.0), 26% glycerol, 7% to 10% PEG 1450, 80 mM $MgSO_4$, 0.02% n-dodecyl- β -D-maltoside, 20 mM NaCl, and 10 mM $CaCl_2$ for native PSII (Cl^- -PSII) (26). For PSII with Br or I substitution, 20 mM NaCl and 10 mM $CaCl_2$ in the crystallization buffer were replaced by 20 mM NaBr and 10 mM $CaBr_2$ or by 20 mM NaI and 10 mM CaI_2 , respectively. Crystallization was carried out in complete darkness at 20 °C. Crystals grew to a full size of $1.0 \times 0.5 \times 0.1$ mm in 3 to 5 days, and the crystals were harvested and transferred to a cryoprotectant solution as described previously (15, 26).

with NaBr and CaBr₂ or with NaI and CaI₂, replacing NaCl and CaCl₂, for Br-PSII and I-PSII, respectively. The crystals were flash-cooled in a nitrogen gas stream at 100 K.

X-Ray Diffraction Measurements and Structure Analysis. X-ray diffraction experiments were performed at BL41XU of SPring-8 (34) at 100K. The wavelengths used were 0.9 Å for Cl-PSII and Br-PSII and 1.0 Å for I-PSII. Diffraction patterns were recorded with a CCD detector Quantum 315 (ADSC) every 0.6° with an exposure time of 4 sec and an x-ray flux of $1.0\text{--}1.8 \times 10^{11}$ photons/sec. The diffraction data were processed with HKL2000 (35).

The structures of PSII with Br⁻ substitution and I⁻ substitution were solved by the molecular replacement method using as a search model a PSII monomer that was generated from the 3.0-Å structure (PDB code: 2AXT) (17), with the program AMORE from CCP4 suite (36). The refinement processes were carried out using Program CNS version 1.2 (37). Rigid-body refinement was performed first to optimize the position of the 2 PSII monomers in the asymmetric unit; based on that refinement, the non-crystallographic symmetry (NCS) matrices were defined and used in the following refinement process. The initial model was improved using simulated annealing refinement to reduce the search-model bias. The structural models were modified according to the electron density map using the graphic program Coot (38). The locations of iodine and bromine atoms were defined using their anomalous Fourier maps. The $F_o - F_c$ omit maps calculated from the refined PSII structures without Br or I atoms were generated with the program CNS version 1.2. The model geometry and quality statistics were calculated with the PROCHECK program (39).

Measurement of Oxygen-Evolving Activities. Oxygen evolution was measured with a Clark-type oxygen electrode at 30 °C under continuous, saturating light

with 0.5 mM phenyl-*p*-benzoquinone and 0.5 mM potassium ferricyanide as electron acceptors. To remove Cl⁻ from PSII samples, the samples were diluted with a Cl⁻-free medium containing 20 mM MES (pH 6.0) and 5 mM CaSO₄ (for supplying Ca²⁺ to PSII) at a concentration of 1 mg chl/mL, mixed with the same volume of precipitation buffer containing 20 mM MES (pH 6.0), 26% PEG 1450, and 5 mM CaSO₄. After incubation for 30 min, the PSII samples were precipitated by centrifugation at $15,000 \times g$ for 15 min and were re-suspended in the Cl⁻-free medium. This washing step was repeated, and the final PSII samples were suspended in 20 mM MES (pH 6.0) at 5 mM CaSO₄ at a concentration of 0.5 mg chl/mL. For measuring the dependency of oxygen evolution on concentrations of Cl⁻, Br⁻, or I⁻, samples were incubated with NaCl, NaBr, or NaI at indicated concentrations for 30 min in the dark on ice, and the activity was measured in the presence of the same concentrations of NaCl, NaBr, or NaI. For measuring the recovery of oxygen evolution of PSII when I⁻ was replaced by Cl⁻ or Br⁻, the samples with I⁻ substitution were washed twice with 20 mM MES (pH 6.0) and 5 mM CaSO₄ and were suspended in the same medium at a concentration of 0.5 mg chl/mL. The samples were supplemented with NaCl or NaBr at indicated concentrations and were incubated in the dark for 30 min. Then oxygen evolution was measured in the presence of the same concentrations of NaCl or NaBr.

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