Insights into Photosystem II from Isomorphous Difference Fourier Maps of Femtosecond X-ray Diffraction Data and Quantum Mechanics/Molecular Mechanics Structural Models

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ABSTRACT: Understanding structure–function relations in photosystem II (PSII) is important for the development of biomimetic photocatalytic systems. X-ray crystallography, computational modeling, and spectroscopy have played central roles in elucidating the structure and function of PSII. Recent breakthroughs in femtosecond X-ray crystallography offer the possibility of collecting diffraction data from the X-ray free electron laser (XFEL) before radiation damage of the sample, thereby overcoming the main challenge of conventional X-ray diffraction methods. However, the interpretation of XFEL data from PSII intermediates is challenging because of the issues regarding data-processing, uncertainty on the precise positions of light oxygen atoms next to heavy metal centers, and different kinetics of the S-state transition in microcrystals compared to solution. Here, we summarize recent advances and outstanding challenges in PSII structure–function determination with emphasis on the implementation of quantum mechanics/molecular mechanics techniques combined with isomorphous difference Fourier maps, direct methods, and high-resolution spectroscopy.

Photosystem II (PSII) is the only known protein complex capable of catalyzing direct solar water-splitting into O₂, protons, and electrons.1–4 Understanding its structure–function relations can provide fundamental insights, essential for the design of artificial photosynthetic systems. The oxygen-evolving complex (OEC) of PSII is a CaMn₄O₅ oxo-manganese cofactor that binds substrate water and splits it into O₂, protons, and electrons through a stepwise catalytic cycle (Figure 1). During each turn of the cycle, the OEC evolves through five “storage” states, Sₙ (n = 0–4), where S₀ is the dark stable state while S₁ and S₄ are the most reduced and oxidized forms, respectively.5,6

The detailed analysis of the catalytic reaction has been challenging and benefited from an interdisciplinary approach, including computational modeling, crystallography, and spectroscopy,7 combined with mutagenesis and kinetic studies. Major breakthroughs in protein X-ray crystallography have resolved the PSII crystal structure from 3.8 Å, to the highest 1.9 Å resolution (PDB accession number 3ARC/3WU2),8,9 establishing the architecture of the OEC and its ligation scheme. A challenge has been that even at the highest resolution, the X-ray dose used for X-ray data collection caused reduction of the OEC,8b consistent with previous studies based on extended X-ray absorption fine structure (EXAFS) spectrometry.10–12

In recent years, X-ray free electron laser (XFEL) pulses with different approaches applied to serial femtosecond crystallography (SFX)13–17 have enabled collection of PSII diffraction data on the femtosecond scale before the onset of significant radiation damage. Several developments emerged from the analysis of XFEL data, and direct comparisons were made to computational structural models. Here, we focus on insights into the PSII machinery obtained from isomorphous difference Fourier methods in crystallography combined with direct methods and quantum mechanics/molecular mechanics (QM/MM) calculations.18–22

Isomorphous Difference Fourier Methods: The S₁ to S₂ Transition. A fundamental limitation of X-ray crystallography is that only the intensity of the scattered radiation is measured, I(h) = |F(h)|², with the structure factor

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Isomorphous difference Fourier methods\textsuperscript{24,25} have important advantages relative to calculations of electronic density differences obtained from refined structural models, allowing for analysis of small changes between an isomorphous pair of structures, \textit{i.e.}, structures with the same cell parameters, orientation, and overall conformation of the system in the unit cell, except for small differences deliberately introduced such as the light-induced \textit{S}\textsubscript{1} to \textit{S}\textsubscript{2} transition. Contrary to eq 2, isomorphous difference methods compute electron density difference maps (as the Fourier transform of the difference of observed amplitudes) with calculated phases for only one of the models:

\[
\Delta \rho(r) = \sum \frac{||F_{\text{obs}}(\text{II})|| - ||F_{\text{obs}}(\text{I})|| e^{i2\pi \mathbf{hr}}}{e^{-2\pi \mathbf{hr}}} 
\]

Equation 2 requires calculation of phases \(\alpha_\text{II} \) and \(\alpha_\text{I} \) for both models and gives the difference of electronic densities as obtained from the refined models. The difficulty with such an approach when applied to very similar structures is that real features in the observed intensity data are not sufficiently distinct from those due to the model bias inherent in the calculation of the phases. Remarkably, such an approximation over the phases gives a very accurate description of the exact map of electron density differences, even when the phases dominate the Fourier transform.

The isomorphous difference Fourier method was initially developed to analyze small ligand binding (comparable in size to a water ligand) such as azide or cyanide binding to heme proteins even when direct Fourier maps were unable to identify the underlying subtle structural changes at relatively low resolution.\textsuperscript{25–28} In its original application, experimental phases were available.\textsuperscript{25–28} In the absence of experimental phases, model phases are used (eq 3) as in the study of Kern et al. where structural changes in the OEC during the \textit{S}\textsubscript{1} to \textit{S}\textsubscript{2} transition were explored for XFEL data obtained at 5.9 Å resolution.\textsuperscript{14,15} We used high-resolution model phases followed by 3-crystal 6-fold noncrystallographic symmetry (NCS) averaging\textsuperscript{18} as described in the Supporting Information of ref [Page 398]
produce a distinct positive peak in the simulated OEC,18 from Mn(III) to Mn(IV), inducing a contraction of the transition involves oxidation of the dangling Mn (Mn4) in the $\text{D170}^-$ before the onset of significant radiation damage. However, simulations of EXAFS spectra based on the coordinates of the model show that they are not fully consistent with EXAFS data for the S1 state because of lack of accuracy in the position of the O atoms of $\mu$-oxo bridges next to the strongly scattering Mn and Ca ions.20 Historically, accurate detection of light O atoms next to metal centers has been challenging. The absence of the O4 $\mu$-oxo bridge in the 3.3 Å resolution structure of PSII32 is an example analogous to the missing carbon atom in the MoFe center of nitrogenase at 1.55 Å resolution prior to the 1.16 Å resolution structure. The difficulty is partly due to ripple effects induced by premature Fourier series termination for data with insufficient (nominal and spatial) resolution. In hindsight, once the presence of the ligand is established, the $F_o$–$F_c$ maps can be used to confirm the location of light ligands, although the accuracy of this method is still limited by the tailing effect of heavier metal ions.

**Isomorphous Difference Fourier Maps Modified for a Varying Unit Cell: S4 State.** The S4 state of PSII has been studied by some of the most recent XFEL experiments16 because it is one of the most important intermediates in the catalytic cycle, detectable right before O–O bond formation and reduction of the OEC. The comparative analysis of the S1 and S4 electron density maps has been challenging, with the two states obtained at different nominal resolution of 5.0 and 5.5 Å, respectively. The challenges are due to (i) the different kinetics between aqueous solution and individual microcrystals, which may have prevented PSII in crystals from fully achieving the S4 state, and (ii) changes in the crystallographic unit cell that may have increased the extent of nonisomorphism.

It has been necessary to modify the isomorphous difference Fourier method and apply it by computing two sets of electron density maps, because the original method was not applicable to analyze structures with different unit cells. Only paired and intensity-scaled reflections were used in reciprocal space for each unit cell, with only one set of model phases.35,34 The resulting maps were realigned by using least-squares procedures (LSQ), as implemented in the Rave package, and back Fourier-transformed to generate new sets of observed amplitudes ($F_{o}^{\text{LSQ}}$) for calculation of difference Fourier map as follows:

$$\Delta F_{o}^{\text{LSQ}} = \sum_i \left( ||F_{o}^{\text{LSQ}}(I)| - |F_{o}(I)|| \right) e^{i\phi_{\text{model}(I)}} e^{-2\pi i(\vec{G} \cdot \vec{r})}$$

To minimize the Fourier series termination effect, only the reflections present in both data sets with 5.0 and 5.5 Å resolution were included in the analysis. The resulting spatial
resolving was limited by the lowest resolution of the compared structures. The analysis based on realigned difference Fourier maps provided valuable insights on the expansion of the CaMn₄O₅ cluster (Figure 3), during the S₂ to S₃ transition, likely induced upon binding of substrate water molecule to the dangling Mn as shown by QM/MM calculations. Figure 3 shows the difference Fourier maps, obtained according to eq 4, based on the rescaling to SFX data sets from the S₁ and S₃ states of PSII. It reveals significant changes in the Fourier maps localized at the OEC. The electron density differences at the OEC are consistent with the QM/MM models, as corresponding to an expansion of the CaMn₄O₅ cluster during the S₁ to S₃ transition.

The QM/MM rearrangement is consistent with binding of a water molecule through a “carousel” mechanism, suggesting that some fraction of the PSII in microcrystals must have advanced beyond the S₂ state. Considering that the S₁ to S₂ conversion resulted in a positive peak inside the OEC near Mn₄O₅ (Figure 2), the net negative peak observed during the S₁ to S₃ transition suggests that the S₂ fraction must have over-compensated whatever fraction of the S₁ was formed. Interestingly, because the metal cluster expands upon S₂ to S₃ oxidation and the cluster contracts during the S₁ to S₃ conversion, it is conceivable that the S₁ to S₃ oxidation might indicate binding of a water molecule, as suggested by various computational models.

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Computational Noise in XFEL Data Sets. Approximations and statistical inferences of the algorithms used for data reduction often introduce nonrandom noise to the experimental data. The analysis of how the resulting computational noise affects the distribution of X-ray scattering intensities is important because noise can scramble weak structural signals of crystallographic data sets (Figures 4 and 5).

A classic example of computational noise concerned the treatment of negative and weak intensity measurements that concluded with a Bayesian statistical solution formulated by French and Wilson (1978). The probability of measuring the intensity of any reflection as negative is known, although sometimes disregarded. Proper processing of negative intensities, however, is critical for the quality of the data and XFEL maps produced from microcrystals. Discussions on negative intensities can be found elsewhere. Here, we limit the presentation to the simplest example showing why it is important to include negative intensity measurements during an intensity merging process. If one measures a systematically absent Bragg reflection (with null average intensity) several times, it is likely that after background subtraction half of the measurements will be positive and half negative. If the five negative measurements are disregarded, arguing that the intensity (i.e., amplitude square) must be positive, the mean value of the reflection would be positive instead of null. Thus, this reflection would be no longer absent.

Improper treatment of negative and weak intensity measurements could lead to various skewed intensity distributions, detectable by statistical tests. With properly processed data sets, the probability density of any given acentric Bragg reflection with intensity between I and I + dI follows the single exponential function, \( \exp[-1/(I)] \), known as the Wilson intensity distribution, where \( \langle I \rangle \) is the mean intensity of all noncentric Bragg reflections. The probability density of fractional intensity differences between pairs of Bragg reflections selected locally in reciprocal space, \( L \equiv (I_1 - I_2)/[(I_1 + I_2)/2] \), provides the L-test because the first and second moments \( \langle LL \rangle \) and \( \langle L^2 \rangle \) are 1/2 and 1/3 for untwinned acentric data, respectively. If twinning occurs, as observed in crystal lattices where the unit cell can adopt more than one orientation, the L moments are lowered and reach a value of 3/8 and 1/5 for fully twinned data.

The skewed intensity distribution identified by the L-test quantifies the level of computational noise.

When twinning is physically impossible, as for the PSII crystals with \( P2_1\bar{2}1_2 \) symmetry, the skewed intensity distribution identified by the L-test quantifies the level of computational noise introduced in the data sets. Figures 4 and 5 shows that an outstanding challenge in the field is the reduction of computational noise in the XFEL data sets of PSII.
structures.45 In particular, Figure 4 analyzes data sets processed with the CrystFEL, cctbx.xfel, and mosflm/XFEL-specific programs, which have been used to process the XFEL PSII data deposited in the PDB.42,46−49 Figure 4a shows that the 4TNJ PSII data set has a much higher level of computational noise than the 4PBU (or other PSII) data sets, processed using the CrystFEL program (or other programs).13−17 When the same L-tests are extended to XFEL data sets from other proteins, it is clear that the problem is not related to the PSII crystals but rather to a data-processing artifact specific to cctbx.xfel (Figure 4b).42,50

Figure 4 analyzes the cctbx.xfel-processed data as divided into three resolution ranges: (i) below 5 Å, (ii) 5−4 Å, and (iii) 4−3 Å. The L-tests were performed on each of the three blocks of data, independently. The 4−3 Å range exhibits the highest level of computational noise because the average intensities are the lowest (Figure 4c). Consistently, Wilson plots (i.e., logarithm of mean intensity in a given resolution shell versus the reciprocal resolution squared) for data sets processed with cctbx.xfel are displaced upward, at higher resolutions, relative to those obtained with CrystFEL from the same crystals (Figure 4d). It is thus concluded that cctbx.xfel has systematically overestimated the intensities of weak reflections, as also acknowledged by the authors of cctbx.xfel.51

In fact, recent inclusion of negative intensities after background subtraction by reprocessing XFEL data sets of the synaptotagmin-1/SNARE complex has partly alleviated the unusual behavior of the L-test and made the XFEL data set comparable to that of the synchrotron data set.51

Analysis of the Most Recent XFEL Structures. Shortly after submission of this manuscript, a new XFEL experiment was reported with the analysis of PSII in the dark stable S1 state (5KAF, 3.0 Å resolution), the S3 state (5TIS, 2F or two-flashes state, at 2.25 Å), and ammonia-bound S3 (5KAI, 2F-NH3 state at 2.8 Å),52 because NH3 is analyzed as an analog of water. The corresponding 5KAF and 5KAI data sets were collected using the same wavelength and experimental setup, having practically the same unit cell parameters (i.e., the maximal cell parameter difference between them is 0.5 Å or 0.25%, smaller than the variations of cell parameters of about 3 Å reported for the two structures).52 Thus, the 5KAF and 5KAI isomorphous pair is suitable for analysis based on the classic isomorphous difference Fourier method. As a control, we calculated isomorphous difference Fourier maps at 2.1 and 3.0 Å resolution between the 4IL6 and 3ARC/3WU2 data sets, using the model-derived phases of 3ARC/3WU2,8a,53 where 4IL6 represents the data set of the Sr-substituted OEC in the presence of dimethyl sulfoxide (DMSO or DMS) and 3ARC/3WU2 represents the structure of wild-type Ca-containing enzyme in the presence of glycerol. The 3ARC/4IL6 pair is ideally suited for a control experiment because it has amplitude differences as a function of reciprocal resolution that are similar to those of the 5KAF/5KAI pair (Figure 5c). The 3ARC/4IL6 has an overall amplitude difference of 31.3% at 3.0 Å resolution (and 35.9% at 2.1 Å

Figure 4. L-test plots for selected SFX data sets. (a) L-test results for data sets of PSII XFEL data are compared for 4PBU (processed with CrystFEL), 4TNJ (cctbx.xfel), and 4UB8 (mosflm/scala). (b) L-test results for data sets obtained for proteins other than PSII. Data processed using cctbx.xfel (4QX0 and 4QX2) appear to be maximally twinned, whereas data processed from the same crystals using CrystFEL (4QX1 and 4QX3) do not. (c) The cctbx.xfel-processed 4QX0 data set is divided into three resolution ranges for L-test: (i) below 5 Å (orange), (ii) between 5 and 4 Å (blue), and (iii) between 4 and 3 Å (black). (d) Comparison of the Wilson plots (logarithm mean intensity versus inverse resolution squared) obtained from two cctbx.xfel processed data sets (black/4QX0 and green/4QX2) and two otherwise similar data sets processed using CrystFEL (red/4QX1 and blue/4QX3) from similar crystals.
resolution), while that for the SKAF/SKA1 pair is 28.7% at 3.0 Å resolution (Figure 5c). The maximal change in fractional unit cell parameters for the 3ARC/4IL6 is 0.33%, which is only slightly larger than the 5KAF/SKA1 pair (0.25%).

In the 3ARC/4IL6 difference-Fourier maps, the largest positive peak observed at $+7.4\sigma$ pinpoints the replacement of a glycerol molecule (GOL/b738) with a DMSO (DMS/b628) molecule plus an ordered water molecule (d567) (Figure 5d). Corresponding 2Fo(Ca/GOL)-Fo(Sr/DMSO) maps and 2Fo(Sr/DMSO)-Fo(Ca/GOL) maps have also unambiguously confirmed this replacement.

The second largest peak is associated with the substitution of Ca by Sr at the OEC (Figure 6c) because Sr is larger and denser than Ca. Upon Sr substitution, the OEC slightly expands, and three of the four Mn centers exhibit significant peaks above $\pm 4\sigma$ due to subtle displacements. Noticeably, Cl-A680 also has a significant negative peak on it, implying a possible loss of occupancy for this Cl$^-$ anion (Figure 6c).

In contrast to the analysis of the Sr substituted PSII, the isomorphous difference Fourier maps between SKAI and SKAF calculated at various resolutions (using model phases retrieved from the PDB), exhibit no significant detectable difference at the $\pm 2.5\sigma$ contour level at the OEC site, nor at $\pm 4\sigma$ in its vicinity (Figure 6ab), suggesting no detectable NH$_3$ binding.

STIS is the first PSII XFEL data set collected at room temperature with sufficiently high resolution for reliable Wilson-plot analysis (Figure 5). However, even for this data set, the L-test (Figure 5a) shows significant deviations from the expected straight line, similarly to the L-test of all of the PSII X-ray data sets deposited in the PDB that have been analyzed by using cctbx.xfel. Furthermore, the mean intensity (Figure 5b) does not exhibit the proper decay and is higher than expected. Therefore, it remains a major challenge to determine whether there is sufficient signal to be extracted in individual XFEL images at high resolution, particularly when detectable X-ray photons might be less than 1 per pixel. In that case, the proper way to obtain accurate estimates of intensities would be to use tens of thousands of individual images to enhance very weak signals with statistically sound treatments of negative and weak intensity signals. Otherwise, as the resolution increases, the mean intensity at high resolution would approach the asymptotic value of the lowest detectable photon numbers per pixel.$^{54}$

**Direct Methods.** Direct methods infer information about phases from measured intensities,$^{55-58}$ exploiting constraints between the phases of different Fourier components due to the atomicity of the molecules and the fact that the electron density should be zero or positive at any point of the unit cell. For
example, phases for small molecules and medium-sized molecular structures can be directly retrieved from accurately measured amplitudes, using the Hauptman–Karle algorithm.\textsuperscript{57,58} However, that method is often not particularly useful when applied to large proteins because the probability of phase relationship is often too broad, and the overall B-factor for protein data sets can be as much as 10 times larger than for small molecules, making the electron density function for atoms very broad and phase retrieval very challenging. To overcome the broadening of the electron density distribution around atoms, one can use normalized structure factors, resulting in point-atoms in maps. To alleviate the ripple effects of premature Fourier series termination, Wilson-expected values are used to fill in missing high-resolution terms.\textsuperscript{59}

Direct methods have suggested that conventional X-ray diffraction data sets, based on an analysis of the 3ARC data,\textsuperscript{8a} exhibit extensive oxidative modifications (Figure 7).\textsuperscript{59} Figure 7 shows, for example, an Ala residue that has been converted into Ser (A283S).\textsuperscript{59} Analogous changes are observed in about 10% of the residues (i.e., at least 538 residues out of the total of 5351 have been modified). The radiation-induced oxygen insertion in amino acids and proteins has been studied for over a century using a variety of biophysical methods (see ref\textsuperscript{59} for a brief historical review, including two recent specific studies on...
oxidation of PSII protein residues\(^{60,61}\). Radiation splits an \(H_2O\) molecule into a highly reactive hydroxyl radical and a highly reductive hydrated electron. The hydroxyl radical is thought to be responsible for the observed oxygen insertion chemistry, while the hydrated electron is responsible for reduction of metal centers such as Mn at the OEC.\(^{8a}\) We note that Fe is not significantly reduced in the SEJX crystal structure because the \(\text{Fe} - \text{O}\) bond distance is 1.75 Å, only slightly elongated relative to the corresponding bond length of 1.73 Å in a low-dose synchrotron structure (code 3M23).\(^{16}\) However, while an elongated Fe–O bond distance would confirm reduction by free electrons, the lack of elongation cannot rule out radiation damage.

We note that the peaks interpreted as additional oxygen atoms were relatively small because only the second half of the data set exhibits protein modifications. These specific fingerprints, in only part of the diffraction data, are nevertheless quite distinct relative to nonspecific damage that usually reduces the diffraction intensity of the entire data set.

| ONGOING CHALLENGES |
|---------------------|
| Other outstanding challenges include an ongoing debate about the possibility of radiation damage during XFEL experiments. In addition to room-temperature SFX, the two highest-resolution SFX data sets for PSII have been recorded by using multiple shots per frozen crystal at liquid N\(_2\) temperatures.\(^{17}\) While the use of cryogenic temperatures has been a common methodology for conventional crystallographic data sets (e.g., for the highest-resolution data set of PSII reported\(^{59}\)), there is evidence that the diffusion rate of hydroxyl radicals generated by radiation might be faster than the rate of sample translation for bringing unexposed parts of the sample to the exposing position. As a consequence, oxygen atoms might be added to many of the protein residues, as observed in cytochrome \(c\) peroxidase.\(^{59,62}\) |

| There is evidence that the diffusion rate of hydroxyl radicals generated by radiation might be faster than the rate of sample translation. |

The self-amplified self-emission process represents another XFEL challenge because the process is highly stochastic with large fluctuations.\(^{63}\) Femtosecond pulses of XFEL are thus quite different from one another, in terms of their intensity profiles. Usually, the effect of such variation is not included in SFX modeling theories.\(^{64-67}\) Therefore, it is conceivable that the SFX data might have recorded a destruction process of molecules in the crystal lattice and that such a destructive process may differ from one XFEL pulse to another, partially explain why merging statistics of XFEL data sets does not always provide significant statistical improvements.\(^{68,69}\)

| CONCLUSIONS |
|--------------|
| We have reviewed recent advancements in the field of PSII structural characterization, with emphasis on outstanding challenges and insights from isomorphous difference Fourier maps of femtosecond X-ray diffraction data as well as direct methods in conjunction with QM/MM structural models. The crystal structure of PSII obtained at 1.9 Å resolution using conventional X-ray crystallography\(^{8a}\) represents a milestone achievement that has confirmed and established many structural features of PSII. However, it is now clear that the reported data reflect X-ray radiation-induced modifications.\(^{8a}\) Similarly, the SFX structure at 1.95 Å resolution is a significant breakthrough, although several challenges remain to be addressed with respect to the analysis of data collected for SFX structures. In particular, the quality of the overall data statistics and the methods for data processing remain |

Figure 7. Direct-methods generated E-map showing conversion of Ala to Ser in the PSII 3ARC data set. The E-map was contoured at +8.0\(\sigma\) (cyan), + 5.0\(\sigma\) (blue), and +2.0\(\sigma\) (green) followed by an interpretation of Ser residue in three different rotameric positions (green, pink, and light red). Adapted with permission from ref 59. Copyright 2016 Wiley.
challenging. We have reviewed some historical literature on statistically sound approaches that might provide valuable solutions to improve both accuracy and consistency of XFEL data sets.65,69 The crystallographic community has decades of experience on how to properly process still diffraction images, for example, in Laue diffraction.70 Like SFX diffraction experiments, one-shot-per-crystal determination using Laue diffraction has also been developed.71 In the next few years, achieving atomic resolution of the OEC for the various S-state intermediates would also require overcoming the variable initial S-state composition due to extensive dark adaptation prior to data collection as well as the intrinsic uncertainty in the positions of oxygen atoms due to weak diffraction of oxygen atoms next to the heavier manganese centers. The resulting structural information should be particularly valuable for understanding structure–function relations of PSI that could inform the development of biomimetic photocatalytic systems.

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**Notes**

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