Conformational substates in different crystal forms of the photoactive yellow protein—Correlation with theoretical and experimental flexibility

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Abstract

The conformational changes during the photocycle of the photoactive yellow protein have been the subject of many recent studies. Spectroscopic measurements have shown that the photocycle also occurs in a crystalline environment, and this has been the basis for subsequent Laue diffraction and cryocrystallographic studies. These studies have shown that conformational changes during the photocycle are limited to the chromophore and its immediate environment. However, spectroscopic studies suggest the presence of large conformational changes in the protein. Here, we address this apparent discrepancy in two ways. First, we obtain a description of large concerted motions in the ground state of the yellow protein from NMR data and theoretical calculations. Second, we describe the high-resolution structure of the yellow protein crystallized in a different space group. The structure of the yellow protein differs significantly between the two crystal forms. We show that these differences can be used to obtain a description of the flexibility of the protein that is consistent with the motions observed in solution.

Keywords: essential dynamics; photoactive yellow protein; photocycle; X-ray crystallography

The photoactive yellow protein (PYP) is a water-soluble photoreceptor protein, first isolated from *Ectothiorhodospira halophila* (Meyer, 1985; Meyer et al., 1987). The protein appears to be involved in a phototactic response of this bacterium to blue light (Sprenger et al., 1993). It binds a chromophore, *p*-coumaric acid, which gives the protein its characteristic yellow color, via a thioester linkage to its unique cysteine (Baca et al., 1994; Hoff et al., 1994a). PYP can be activated with light, to go through a photocycle consisting of at least four to five transient intermediates. The ground state (pG) has a UV/VIS absorbance maximum at 446 nm. After absorption of a blue photon, the protein goes through a primary excited state and first transient ground state, into a strongly red-shifted intermediate (Ujj et al., 1998) at the ps time scale (Meyer et al., 1991; Baltuska et al., 1997; Chosrowjan et al., 1997; Chagenet et al., 1998). A more moderately red-shifted intermediate (pR), absorbing maximally at 465 nm, is formed on the ns time scale (Hoff et al.,

1994b). The pR intermediate spontaneously converts into a blue-shifted intermediate (pB; absorbing maximally at 355 nm) at the sub-ms time scale (Meyer et al., 1987; Hoff et al., 1994b). The pB intermediate subsequently relaxes back to pG on a subsecond time scale (Meyer et al., 1987, 1991; Hoff et al., 1992, 1994b), or faster, in a light-dependent reaction (Miller et al., 1998). These transitions are accompanied by a double-bond isomerization of the *p*-coumaryl chromophore of PYP (Kort et al., 1996; Xie et al., 1996; Genick et al., 1997, 1998; Perman et al., 1998).

The structure of the ground state of PYP (i.e., pG) has been solved to 1.4 Å, and subsequently to 0.82 Å resolution (Borgstahl et al., 1995; Genick et al., 1998), revealing the intimate contacts that the chromophore makes with specific residues lining a pocket inside the protein. Subsequent studies, using Laue diffraction and cryo-crystallography, have revealed what happens during and after chromophore isomerization (Genick et al., 1997, 1998; Perman et al., 1998). These studies suggest that the conformational changes in PYP are small and limited to the immediate environment of the chromophore. In contrast, for PYP in solution, formation of the blue-shifted intermediate pB is paralleled by a major conformational transition that carries typical features of a partial protein unfolding event, as reported by UV/VIS and FTIR spectroscopy, mass spectrometry, and ¹H-NMR analyses (van Brederode et al., 1996; Rubinstenn et al., 1998; Hoff et al., 1999). This raises the question as to what extent the conformational changes and dynam-

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ics of PYP are influenced by the constraints of the crystal lattice. In an attempt to address this issue, we describe the 1.39 Å crystal structure of the PYP ground state in a different crystal form than that reported previously (Borgstahl et al., 1995). The conformational changes in the protein in the different crystal lattice environments are analyzed and compared with concerted motions calculated from computer simulations and NMR structures. Although the conformation of PYP appears to be affected by the interactions within the crystal, we show that these conformational changes are not “accidental” and can give useful information about the flexibility of the protein, and possibly the structural events during the photocycle.

Results

Essential dynamics of PYP

A previous study has provided an initial description of chromophore-linked protein motions of PYP in solution (van Aalten et al., 1998b). Here we include new experimental data from NMR and crystal structures. Three different ensembles of PYP were studied by essential dynamics, the previously published MD simulation (van Aalten et al., 1998b), a new ensemble generated with the CONCOORD program using a crystal structure of PYP, and the NMR ensemble of structures described in Protein Data Bank (PDB) entry 3PHY. As described in Materials and methods, only backbone atoms in secondary structure elements were included in the definition of the covariance matrix. A direct way of comparing the concerted motions resulting from these three calculations is by projecting the eigenvectors of one set onto those of another set. The three eigenvectors with the largest eigenvalues (i.e., those that together describe a large part of the total motion in the ensemble, typically 40–60%) of one set were taken and projected onto the first 25 eigenvectors, which are taken to represent the “essential subspace” (Amadei et al., 1993), of the other sets. Table 1 shows that the first 25 eigenvectors (out of 825) from one set are sufficient to reproduce the motions described by the three most significant eigenvectors of another set by about 50–80%. This is comparable to previous experiments and demonstrates a significant similarity between the concerted motions calculated from the three different ensembles of structures. Thus, this allows a direct comparison between concerted motions calculated from experimental data (the NMR structures) and those derived from theoretical calculations. We will attempt to use these eigenvectors to describe the confor-

mational change between two different crystal forms of PYP using only a few variables.

PYP in space group $P6_5$

PYP crystals were obtained in space group $P6_5$ ($P6_5$ -PYP) and solved to 1.39 Å resolution. The structure was refined to a final R -factor and R -free of 0.139 and 0.198, respectively, with no σ cutoff on the data and good geometry. The structure appears to be well ordered apart from residues 1–3 and 114–116 (see Materials and methods). The root-mean-square deviation (RMSD) of the $C\alpha$ atoms after superposition of $P6_5$ -PYP and the previously reported PYP structure in space group $P6_3$ (Borgstahl et al., 1995) ($P6_3$ -PYP) is 0.48 Å [estimated mean coordinate error estimated from a Luzatti plot (Luzatti, 1952) is 0.05 Å]. Comparison of our $P6_5$ -PYP structure to $P6_3$ -PYP reveals significant differences (Fig. 1). The largest differences are found around residue 20, which is in a surface loop that is not well defined in the NMR structure (Dux et al., 1998) and is mobile in MD simulations (van Aalten et al., 1998b). Residues 80–90 correspond to the end of α -helix 5, which, together with the loop around residue 100, also appeared to be flexible during MD simulations (van Aalten et al., 1998b).

The different packing environments between the two crystal forms are illustrated in Figure 2. It appears that there are more intimate contacts in the $P6_3$ -PYP crystal than in our $P6_5$ crystal form. Although the patterns of distances to symmetry related molecules are similar, there is a trend for those distances to be larger in $P6_5$ -PYP. For example, with distances of less than 3.5 Å defined as contacts, $P6_3$ -PYP has 58 crystal contacts with symmetry-related molecules, and $P6_5$ -PYP only 39. Accessibility calculations with WHAT IF (Vriend, 1990) show that 837 Å² of protein surface is buried by symmetry-related molecules in the $P6_3$ -PYP crystal, whereas only 586 Å² is buried in $P6_5$ -PYP. Thus, it seems that the protein molecules in our crystal form pack more loosely than in $P6_3$ -PYP. This may explain the disordered residues in our structure, which appeared to be ordered in $P6_3$ -PYP.

The conformational changes described above point to noticeable differences in protein conformation in the two crystal lattice environments. To investigate whether the PYP structure is distorted randomly or whether these differences can be described by motions corresponding to the flexibility of the protein in solution, we compare the conformational change between the crystal forms with the large concerted motions calculated by essential dynamics. Atoms corresponding to those used for the essential dynamics calculations were extracted from the $P6_5$ -PYP and $P6_3$ -PYP structures. Then, a normalized vector (PYPcry) in configurational space was calculated that describes the atomic shifts required to move from the $P6_5$ -PYP conformation to that of $P6_3$ -PYP. We projected the eigenvectors calculated from the NMR and theoretical ensembles (see above) onto this vector, and thus asked to what extent do the essential subspaces (i.e., the first 25 out of the 825 eigenvectors) reproduce the PYPcry vector? Figure 3 shows that using only 25 variables (eigenvectors), the conformational change between the two crystal structures can be described at 40–80% of the total change. Interestingly, the eigenvectors calculated from the theoretical ensembles are able to reconstruct a larger part of the conformational change than the NMR eigenvectors. Figure 3 also shows that a single eigenvector from the CONCOORD ensemble (eigenvector 6) is able to reproduce the conformational change at more than 40% of the total. The motion described by this eigenvector is shown in Figure 4 and is similar to eigenvector motions

Table 1. Comparison of eigenvectors obtained from three different ensembles^a

	MD (3 evs)	CONCOORD (3 evs)	NMR (3 evs)
MD (25 evs)	1.00	0.84	0.58
CONCOORD (25 evs)	0.75	1.00	0.55
NMR (25 evs)	0.53	0.46	1.00

^aThe numbers shown are average square inner products between sets of eigenvectors. They indicate how well the essential space (arbitrarily set to have a dimension of 25 eigenvectors) obtained from one ensemble reproduces the first three eigenvectors (which typically cover 40–60% of the total motion) calculated from another ensemble.

Fig. 1. A: Comparison of α traces. P6₅-PYP (colored by shift in atomic position) is compared with P6₃-PYP (gray). The atomic shifts are colored from blue (0.0 Å) to red (>0.25 Å). The chromophore is shown as a stick model bound to Cys69. Several α positions are labeled. The N- and C-termini are labeled as well. Structures were superimposed using α atoms. **B:** Positional shifts are shown as a bar graph.

observed from PYP MD simulations as reported previously (van Aalten et al., 1998b). In particular, the two short helices (residues 44–50 and 54–57, lower left corner of Fig. 4) that shield the chromophore from the solvent show a hinge bending motion, possibly with the conserved glycine 51 (see Fig. 1A) acting as the hinge.

Discussion

The study of functional protein dynamics is hampered by the absence of an experimental technique that measures large-scale motions in a protein as a function of time. Instead, molecular dynamics simulations have been widely used to approximate this information. Although such computational approaches have yielded clues as to how protein flexibility might be important for function, it has been difficult to verify experimentally the dynamic properties that were obtained. Here we have used the essential dynamics technique (Amadei et al., 1993) to extract and compare large concerted motions from several ensembles of PYP structures. Earlier studies

have indicated that this technique may be applied directly to the ensemble of structures resulting from NMR distance geometry calculations (Abseher et al., 1998; van Aalten et al., 1998a). Here we have also shown that the eigenvectors with the largest eigenvalues calculated from the PYP NMR ensemble show a significant overlap with eigenvectors from MD or CONCOORD calculations. The CONCOORD approach in particular seems promising, as the eigenvectors obtained are similar to those calculated from an MD simulation in water, yet CONCOORD consumed about 350 times less CPU time (30 min vs. 11,000 min for the standard MD simulation on an SGI R10000 195 MHz computer). Thus CONCOORD may allow an accurate prediction of concerted motions in large protein systems in the near future (de Groot et al., 1999).

The crystals of P6₅-PYP described in this study were of a different crystal form than the P6₃-PYP structure reported previously (Borgstahl et al., 1995). This change of space group leads to a significant reduction in intermolecular contacts between the symmetry-related molecules and might also be responsible for the two disordered regions identified in the structures reported here.

Fig. 2. Differences in crystal packing. Two views of P6₃-PYP (top) and P6₅-PYP (bottom) are shown. Atoms are colored by shortest distance to any atom of any symmetry-related molecule using GRASP (Nicholls et al., 1991). Green represents distances <2.7 Å, white represents any distance larger than 4 Å. Shades of green linearly represent distances between 2.7 and 4 Å.

Overall, the change in crystal packing causes a significant conformational change, significantly higher than the coordinate error. The magnitude of this change is at the upper range of discrepancies between nonisomorphous lysozyme crystal structures (average RMSD for C α atoms 0.42 Å) (Zhang et al., 1995), and those observed in an analysis of other proteins whose structures have been determined in various crystalline environments (mean RMSD 0.33 Å) (Chothia & Lesk, 1986). In some cases, crystal packing forces do have a substantial effect on the conformation of a macromolecule, but this has been limited largely to DNA–oligonucleotide structures (Dickerson et al., 1994). For most proteins, the variations are not as dramatic, and in many cases have been limited mainly to loop regions on the surface of the protein. However, rather than dismissing these changes as meaningless crystal packing effects, we have studied the conformational change between two PYP crystal forms in terms of concerted motions calculated from experimental and theoretical ensembles of structures to see if these changes can teach us anything about the flexibility of the molecule. It is apparent that the crystal lattice does not just force some random conformational change onto the molecule, but rather the molecule moves along essential eigenvectors to accommodate the different lattice environment. Thus, an ensemble of crystal structures could be conceived to represent the conformational flexibility of the molecule, and therefore, quite useful, rather than discarding the differences as irrelevant, “pack-

ing effects.” Consequently, it might be possible to calculate eigenvectors directly from such an ensemble of crystal structures. Indeed, initial studies have indicated this may be the case (van Aalten et al., 1997a; de Groot et al., 1998). It should be noted that from their analysis of thermal factors of main-chain atoms of nonisomorphous lysozyme structures, Matthews and colleagues (Zhang et al., 1995) concluded that in some crystal forms main-chain motion may be constrained by crystal contacts and thus would not represent the dynamic behavior in solution. However, this may be a restriction in magnitude or the range of the motion. The direction of these motions may still be consistent with the conformational landscape that a particular protein would adopt. In addition, other differences exist between the two crystal forms, namely solvent conditions and temperature of data collection. However, the end result of extracting useful information on conformational flexibility from the differences between different crystalline environments remains.

Previous cryo-crystallography and Laue-diffraction studies have all used crystals in the P6₃ space group (Genick et al., 1997, 1998; Perman et al., 1998), and showed small conformational changes, limited to the immediate chromophore environment in the photocycle transitions studied. In contrast, for PYP in solution, formation of the blue-shifted intermediate pB is paralleled by a major conformational transition that carries typical features of a (partial) protein unfolding event, as reported by UV/VIS and Fourier transform infrared spectroscopy (FTIR) spectroscopy, mass spectrom-

Fig. 3. Projection of eigenvectors onto the vector PYPcrystal, which describes the conformational change between the P6₅-PYP and P6₃-PYP crystal structures. The first 25 eigenvectors of the NMR, MD, and CONCOORD ensembles are projected onto PYPcrystal and the cumulative square inner product is shown in the figure.

etry, and ¹H-NMR analyses (van Brederode *et al.*, 1996; Rubinstenn *et al.*, 1998; Hoff *et al.*, 1999). These observations are more in line with the presumed signaling function of PYP, which ultimately should result in a negative phototactic response of the bacterium. The conformational flexibility described previously (van Aalten *et al.*, 1998b), and in this study, calculated from computer-generated ensembles, an ensemble of NMR structures, and the differences between two crystal forms, presents evidence for chromophore-linked concerted backbone motions of loops and helices over significant distances. In all eigenvectors, similar regions in the protein are observed to move with respect to each other. The most mobile

parts include the small helical N-terminal domain and the two short helices in the 40–60 region below which the chromophore is buried. MD simulations have also shown these helices to move, in concert with the chromophore itself (van Aalten *et al.*, 1998b). From these studies it appears that the hinge bending motion around the conserved Gly51 may constitute a conformational change involved in PYP signaling. In addition, these helices are part of the consensus region of the PAS domain, which is a family of “sensing domains” (Pellequer *et al.*, 1998).

Thus, the question arises whether the conformational flexibility of PYP in crystalline form in the cryo-crystallography and Laue

Fig. 4. Representation of the motion along eigenvector 6 from the CONCOORD ensemble. Structures projected at -1.5 nm (most transparent) and 1.5 nm (least transparent) are shown. Helices are colored red; strands are colored blue.

diffraction studies could be inhibited. From the data presented here, it is apparent that the structure of PYP can be influenced by interactions within the crystal lattice. It is not unconceivable that if such interactions exist, the flexibility of the protein could be affected as well. To rule out that conformational freedom in these studies is limited because of symmetry contacts, it may be useful to repeat such experiments with PYP in P6₅, which may be able to accommodate somewhat larger (or different) conformational changes than the P6₃ space group.

Materials and methods

Purification, reconstitution, and crystallization

Apo-PYP was purified as described previously (Kroon et al., 1996). Briefly, apo-PYP was expressed in *Escherichia coli* M15/pHis. Lysed cells were dialyzed against 50 mM phosphate buffer, pH 7. The resulting apo-PYP containing solution was used for reconstitution with the chromophore *p*-coumaric acid (4-hydroxy-cinnamic acid) (Kroon et al., 1996). After reconstitution, further purification was achieved with Ni²⁺-affinity chromatography, using the N-terminal poly-histidine tag. This tag was subsequently removed by enterokinase treatment, followed by ion-exchange chromatography. Freeze-dried protein was dissolved in 50 mM phosphate buffer pH 7, passed over a gel-filtration column, and concentrated to 25 mg/mL.

A new PYP crystal form was obtained under conditions different from those reported previously (McRee et al., 1986, 1989; Borgstahl et al., 1995). These crystals were obtained by the vapor diffusion method in which 1 μ L of protein solution was mixed with an equal volume of well solution, containing PEG 2K and 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) pH 6.5. Crystals appeared within three days and grew as hexagonal rods, often with several rods joined at their bases. Larger and more regularly shaped crystals were obtained by micro-seeding, using the same protocol as described above. The crystals were of space group P6₅ with one molecule in the asymmetric unit.

Data collection

Diffraction data were collected at beamline X26C at the National Synchrotron Light Source, Brookhaven National Laboratory, using a 300 mm MAR Research image plate detector. Data were collected at 100 K by freezing crystals in an Oxford Cryosystems cryostream. Diffraction data were integrated and scaled with the HKL package (Otwinowski & Minor, 1997). Further details of the data-collection statistics are presented in Table 2.

Solution and refinement

The PYP structure was solved by molecular replacement with the program AMoRe (Navaza & Saludjian, 1997) using the P6₃ form of PYP (PDB entry 2PHY (Borgstahl et al., 1995), referred to here as P6₃-PYP) as a search model, excluding the chromophore. Using 8.0–4.0 Å data, a single solution was obtained. After rigid body refinement in AMoRe, the *R*-factor was 0.283, with a correlation coefficient of 0.78. Initial refinement was performed with CNS (Brünger et al., 1998) with iterative model building using O (Jones et al., 1991). Progress of the refinement is further detailed in Table 3. After a few cycles of model building and placement of

Table 2. Details of P6₅-PYP data collection^a

Temperature	100 K
Wavelength	1.08 Å
Space group	P6 ₅
Cell dimensions	$a = b = 40.56$ Å, $c = 118.09$ Å
Resolution range	15.0–1.39 Å (1.44–1.39 Å)
Number of observed reflections	194,171
Number of unique reflections	21,796 (2,190)
Redundancy	8.9
$I/\sigma I$	23.1 (21.3)
Completeness	98.8% (100.0%)
R_{merge}	0.039 (0.126)

^aValues between brackets are for the highest resolution shell.

some well-defined water molecules (4σ peaks in $F_{\text{obs}} - F_{\text{calc}}$ maps and at least one hydrogen bond to the protein), the *p*-coumaric acid chromophore could be clearly seen in the $F_{\text{obs}} - F_{\text{calc}}$ maps (Fig. 5) and was built into the model. Further rounds of model building and refinement allowed placement of additional water molecules and assignment of some alternate side-chain conformations (Table 3). Subsequent refinement was performed with SHELX-97 (Sheldrick & Schneider, 1997). After introduction of anisotropic *B*-factors, further alternate conformations and water molecules could be modeled. In the last stages of the refinement, hydrogen atoms were added using the riding-hydrogens model (Sheldrick & Schneider, 1997).

During the course of the refinement residues 1–3 and residues 114–116 appeared to be disordered. The maps showed some evidence of several possible conformations for these regions, and many attempts were made to build these regions in, which accounts for the fluctuations in *R* and *R*-free in Table 3. However, because we could not determine their conformations with a reasonable degree of confidence, they were left out of the model. Both regions have high *B*-factors in the P6₃-PYP structure (Borgstahl et al., 1995). In addition, these regions are underdetermined in the NMR structure of PYP (Dux et al., 1998).

Table 3. Progress of P6₅-PYP refinement^a

Molecular replacement	15.0–4.0 Å, $R = 0.283$
Model building, waters	15.0–2.0 Å, $R = 0.204$, R -free = 0.256
Chromophore, waters	15.0–2.0 Å, $R = 0.191$, R -free = 0.238
Alternate conformations	15.0–1.39 Å, $R = 0.193$, R -free = 0.218
SHELX, anisotropic Bs	15.0–1.39 Å, $R = 0.151$, R -free = 0.221
Riding hydrogens model	8.0–1.39 Å, $R = 0.145$, R -free = 0.205
Final (0.0 σ cutoff)	8.0–1.39 Å, $R = 0.139$, R -free = 0.198
Final (4.0 σ cutoff)	8.0–1.39 Å, $R = 0.138$, R -free = 0.195
Number of atoms	957, 151 waters
Number of SHELX parameters	9,942
RMSD bonds	0.008 Å
RMSD angles	1.92°
Average isotropic <i>B</i>	20.34 Å ²

^aAll *R*-factors are calculated without a σ cutoff on the data, unless stated otherwise. Each step represents several rounds of model building, during which occasionally disordered stretches of residues were omitted (see Materials and methods).

Fig. 5. Stereoview of the electron density around the chromophore. An $F_o - F_c$ map just before including the chromophore is shown in magenta. The $2F_o - F_c$ map after building in the chromophore and subsequent refinement is shown in cyan. σ levels were 1.0 and 2.5 for the $2F_o - F_c$ and $F_o - F_c$ maps, respectively. Figures 1A and 5 were made using MOLSCRIPT (Kraulis, 1991), BOBSCRIPT (Esnouf, 1997), and Raster3D (Bacon & Anderson, 1988; Merritt & Murphy, 1994).

Analysis of PYP dynamics

PYP conformational flexibility was studied using the “essential dynamics” method (Amadei *et al.*, 1993). This method is able to describe protein conformational changes using a limited set of degrees of freedom, calculated from an ensemble of structures (e.g., see van Aalten *et al.*, 1995a, 1998b). In most cases, ensembles of structures calculated from molecular dynamics simulations have been used (van Aalten *et al.*, 1995b, 1996; Peters *et al.*, 1996; Mello *et al.*, 1998). Recently, however, it has been shown that the collection of structures resulting from distance geometry calculations with NMR (Abseher *et al.*, 1998; van Aalten *et al.*, 1998a) or groups of related crystal structures (van Aalten *et al.*, 1997a; de Groot *et al.*, 1998) can also yield such ensembles suitable for essential dynamics analyses. The essential dynamics method is based on the diagonalization of the covariance matrix, which is built from correlations between atomic displacements in the ensemble of structures (van Aalten *et al.*, 1997a). The resulting eigenvectors describe concerted displacements of certain groups of atoms, and the eigenvalues are the amplitudes of these motions. Usually only a few eigenvectors (typically 10–20) suffice to describe almost all conformational substates accessible to the protein. Such a description of large (domain) motions has proved useful in linking dynamic properties to protein function in a number of cases (van Aalten *et al.*, 1995a, 1997b; de Groot *et al.*, 1998; Mello *et al.*, 1998).

Here, we use three different ensembles of PYP structures to describe concerted fluctuations of atoms in the PYP ground state:

- A previously published 400 ps MD trajectory of PYP in water (van Aalten *et al.*, 1998b).
- An ensemble of structures obtained from the P6₃-PYP crystal structure, using CONCOORD (de Groot *et al.*, 1997), a method that generates structures using a novel distance geometry algorithm with empirical constraints on distances between atoms.
- The NMR ensemble of PYP structures (Dux *et al.*, 1998), from the PDB entry 3PHY wherein 26 structures are given that fulfill the distance restraints as determined by the NMR experiment. Thus, provided the experimental data to parameter ratio is sufficient, PYP dynamics can be studied in terms of conformational differences within this ensemble of structures.

To exclude possible errors resulting from conformational flexibility caused by lack of restraints in the NMR experiment, we only included in our analysis areas of the protein that were well determined in both the NMR experiment and the crystal structures. The chromophore and the backbone atoms of secondary structure elements were used, with the further exclusion of the helix starting around residue 20, which is marked as underdetermined in the NMR structure. With these restrictions, 275 atoms were selected, resulting in an 825-dimensional configurational space. Covariance matrices were built from the selected atoms. The eigenvectors with the largest corresponding eigenvalues, resulting from the subsequent diagonalizations, were directly compared to study the agreement between the different methods. Finally, the eigenvectors were used to study the conformational change between the two different PYP crystal forms.

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