Population shift of binding pocket size and dynamic correlation analysis shed new light on the anticooperative mechanism of PII protein

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ABSTRACT

PII protein is one of the largest families of signal transduction proteins in archaea, bacteria, and plants, controlling key processes of nitrogen assimilation. An intriguing characteristic for many PII proteins is that the three ligand binding sites exhibit anticooperative allosteric regulation. In this work, PII protein from Synechococcus elongatus, a model for cyanobacteria and plant PII proteins, is utilized to reveal the anticooperative mechanism upon binding of 2-oxoglutarate (2-OG). To this end, a method is proposed to define the binding pocket size by identifying residues that contribute greatly to the binding of 2-OG. It is found that the anticooperativity is realized through population shift of the binding pocket size in an asymmetric manner. Furthermore, a new algorithm based on the dynamic correlation analysis is developed and utilized to discover residues that mediate the anticooperative process with high probability. It is surprising to find that the T-loop, which is believed to be responsible for mediating the binding of PII with its target proteins, also takes part in the intersubunit signal transduction process. Experimental results of PII variants further confirmed the influence of T-loop on the anticooperative regulation, especially on binding of the third 2-OG. These discoveries extend our understanding of the PII T-loop from being essential in versatile binding of target protein to signal-mediating in the anticooperative allosteric regulation.

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Key words: PII protein; allosteric regulation; signal transduction; population shift; dynamic correlation analysis.

INTRODUCTION

The PII protein, as one of the most widely distributed families of signal transduction proteins, has key functions in terms of regulation of central metabolic processes by sensing signals from the carbon, nitrogen and energy status within the cells.1,2 It is able to regulate the activity of not only key metabolic and regulatory enzymes, but also transcription factors and even transport proteins.3–5 The control of PII protein on nitrogen-storage metabolism is due to the binding of PII protein toward N-acetyl-L-glutamate kinase (NAGK) in cyanobacteria and plants which controls the biosynthesis of arginine.6–8 The binding of PII is a response to the cellular concentration of central metabolites ATP, ADP, and 2-oxoglutarate (2-OG), which reflect the cellular energy, carbon and nitrogen abundance.2,5,9 These metabolites bind to PII in an interdependent manner, thereby transmitting metabolic information into structural states.2,10 Another intriguing characteristic is that the three ATP binding sites and the three 2-OG binding sites each exhibit negative cooperativity, which allows PII to sense a wide range of metabolite concentrations.10–13

As revealed from numerous crystal structures, PII proteins show a highly conserved three-dimensional structure. It is composed of homotrimers with each subunit of 12 to 13 kDa and two ferredoxin-like fold-containing cores (βαβ-βαβ) (Fig. 1).2,14 There is a long and flexible T-loop extending outward from each subunit and it is believed that the flexibility of these T-loops is essential...
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MATERIALS AND METHODS

Structures and systems preparation

Crystal structures of PII protein with different number of bound 2-OG (PDB code: 2XZW) were employed. Residues missed in the original PDB file were added using Modeller 9v27 and the enumeration algorithm28 was used to increase the accuracy of the calculated structure. Three PII protein structures with different states of ligand binding were constructed: PII protein with 2-OG only in the binding pocket of chain A (PII(OG1)); PII protein with 2-OG in both chain A and B (PII(OG2)); PII protein with 2-OG in both chain A, B and C (PII(OG3)). For each case, ATP and Mg2+ exist in each of the subunits.

Each structure was first neutralized by adding sodium counter ions randomly with the xLeap module of Amber 10.029 and then solvated in a rectangular box of TIP3P water molecules30 with a solute-wall distance of 12 Å. The solvated systems were energy-minimized prior to the molecular dynamics simulations. Each system was minimized by six consecutive rounds with each round of 1500 steps. Harmonic constraints were applied to all non-hydrogen atoms with the strength of 500, 400, 300, 200, 100, and 0 kcal mol−1 at each round. After that, the system was slowly heated from 0 K to 300 K in 120 ps. Then the systems were conducted in a NVT ensemble for 50 ps followed by a 100 ps simulation in a NPT ensemble.

Molecular dynamics simulations

The molecular dynamics simulations were performed with the Sander module Amber 10.0 using the force field developed by Cornell et al.31 with a periodic boundary condition in the NPT ensemble at 300 K. Langevin dynamics was used with the collision frequency of 1.0 ps−1 and constant pressure of 1 atm. The Shake algorithm32 was applied to fix all covalent bonds. The Particle Mesh Ewald (PME) method33 was used to treat long-range electrostatic interactions. A residue-based cutoff of 10 Å was applied to the noncovalent interactions. No constraint was applied during molecular dynamics simulations which lasted 1.0 ns for each system to get an equilibrium state. A time step of 2.0 fs was used and the coordinates of the simulated complexes were saved every 1.0 ps.

Binding free energy calculations

The MM-GBSA approach,34 which is implemented in the Amber program, was applied to compute the binding free energy (∆G(binding)) of ligand. The ∆G(binding) was determined according to the following equations:

\[
\Delta G_{\text{binding}} = \Delta G_{\text{polar}} + \Delta G_{\text{nonpolar}}
\]

(1)

\[
\Delta G_{\text{polar}} = \Delta G_{\text{ele}} + \Delta G_{\text{GB}}
\]

(2)

\[
\Delta G_{\text{nonpolar}} = \Delta G_{\text{vdW}} + \Delta G_{\text{SA}}
\]

(3)
where $\Delta G_{\text{polar}}$ is the free energy for the polar interaction; $\Delta G_{\text{nonpolar}}$ is for the nonpolar interaction; $\Delta G_{\text{ele}}$ is the electrostatic interaction free energy; $\Delta G_{\text{GB}}$ is the electrostatic solvation free energy; $\Delta G_{\text{vdW}}$ is the van der Waals interaction energy, $\Delta G_{\text{RSA}}$ is the nonpolar solvation free energy. Default parameters were used to calculate the overall binding free energy and its decomposition at residue level.

**Hydrogen-bond interactions analysis**

Since the molecular dynamics simulations include hydrogen atoms, it is possible to make an analysis of hydrogen-bond properties. The hydrogen-bond interactions, which were performed with the Ptraj module of Amber 10.0, are assumed to be present if the participating heavy atoms are 3.5 Å apart and the angle formed between the heavy atoms and donated hydrogen is larger than 120°.

**Dynamic correlation analysis**

Dynamic correlation analysis was employed to identify residues that mediate the signal transduction during the anticooperative process. This approach is based on the correlation coefficient between each pair of residues obtained from the trajectory of molecular dynamics simulations. The correlation coefficients are calculated according to the following equation:

$$C(R_s, R_t) = \frac{\sum (R_s - <R_s>) (R_t - <R_t>)}{\sqrt{\sum (R_s - <R_s>)^2} \sqrt{\sum (R_t - <R_t>)^2}}$$

(4)

where $C(R_s, R_t)$ is the correlation coefficient between residues $s$ and $t$. $R_s$ and $R_t$ are the coordinates of residues $s$ and $t$; $<R_s>$ and $<R_t>$ are the average coordinates of residues $s$ and $t$ calculated based on the whole trajectory and $N$ is the total number of snapshots recorded in the trajectory during the molecular dynamics simulations.

The residue $m$ that mediates the signal transduction between residues $s$ and $t$ with the largest probability is identified according to the following equation:

$$C(R_s, R_m, R_t) = \max\{C(R_s, R_i, R_t)\} = \max\{|C(R_s, R_i)| \times |C(R_i, R_t)|\}_{i=1, 2, \ldots, N}$$

(5)

where $C(R_s, R_m, R_t)$ is the correlation coefficient between residues $s$ and $t$ mediated by residue $i$; $C(R_s, R_i)$ is the correlation coefficient between residues $s$ and $i$; $C(R_i, R_t)$ is the correlation coefficient between residues $i$ and $t$; $|C|$ indicates absolute value; $N$ is the total residue number of the protein. The residue holding the maximal value of $C(R_s, R_m, R_t)$ is considered to mediate the signal transduction between residues $s$ and $t$ with the largest probability ($C(R_s, R_m, R_t)$). After the first round, residues mediate the signal transduction between residues $s$ and $m$ with the largest probability is to be discovered and the same for residues $m$ and $t$. The procedure is continued until no more residues could be found.

**Overexpression and purification of PII variants**

Site-directed mutagenesis of gltB and cloning into the Strep tag fusion vector pASK-IBA3 (IBA GmbH, Göttingen, Germany) was performed and described previously. The vectors were transformed into *Escherichia coli* strain RB9060 and purified using affinity chromatography as described earlier.

**ITC measurements**

ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal) in buffer containing 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 25 mM NaCl, 10 mM KCl, 1 mM MgCl$_2$ and 1 mM ATP (pH 7.5); 2 mM of the titrant 2-OG were dissolved in the same buffer. After an initial 2 μL injection 6 μL were injected 45 times into the 1.4285 mL cell with stirring at 155 rpm at a temperature of 25°C. The PII concentration in the cell was 33 μM (trimer concentration). The binding isotherms were calculated from received data and fitted to a three-site binding model with the MicroCal ORIGIN software (Northampton, MA) as indicated.

**RESULTS**

**Definition of 2-OG binding pocket size**

Structures of PII trimers with all three sites fully occupied and with one or two bound molecules of 2-OG not only shed first light on structural basis of anticooperativity but also help revealing the anticooperative mechanism from perspectives of dynamics. The population shift of binding pocket size employed here to demonstrate the allosteric effects upon 2-OG binding, is defined by identifying residues that contribute greatly to the binding of 2-OG through decomposition of binding free energy followed by analysis of hydrogen–bond interactions.

It is reported that 2-OG is bound to a Mg$^{2+}$ ion, which is coordinated by three phosphates of ATP, and by ionic interactions with the highly conserved residues Gln39 and Lys58 together with the B- and T-loop backbone interactions. Table I lists the overall binding free energy for structures with different ligand binding states. The slight differences among their overall binding free energy ($\Delta G_{\text{binding}}$) mainly result from the polar interactions ($\Delta G_{\text{polar}}$), namely the electrostatic interaction energy ($\Delta G_{\text{ele}}$) and the electrostatic solvation free energy ($\Delta G_{\text{GB}}$). When examining the energy contribution of each residue [Fig. 2(A)], it can be seen that Arg9,
sequence Phe36-Gly41 of the PII T-loop, Lys58 and Gly87 are the main contributors to the energy differences upon 2-OG binding. Among these residues, Arg9 makes contributions to the binding of the first and third 2-OG although it does not make large contributions when the second 2-OG binds to the binding pocket. Gln39 influences the ligand binding most in the sequence Phe36-Gly41. It is evident that both Lys58 and Gly87 make large and positive contributions to the ligand binding. Therefore, Arg9, Gln39, Lys58, and Gly87 were finally chosen as the residue-level indicators to describe the size of the binding pocket.

Since hydrogen-bond interactions always play an important role for ligand binding, hydrogen-bond interactions analysis is employed to identify atoms that can be used to describe the size of the binding pocket at an atomic level. The analysis was conducted between the residues Arg9, Gln39, Lys58, Gly87, and 2-OG. As shown in Figure 2(B), hydrogen-bonds are formed between the main chain N atom of Gln39 and O1 (O2) of 2-OG, the side chain N atom of Lys58 and O4 (O5) of 2-OG. There are also hydrogen-bonds between the main chain N atom of Gly87 and the O4 (O5) of 2-OG. However, no hydrogen-bond is formed between Arg9 and 2-OG due to their long distance although Arg9 also makes contributions to the binding of 2-OG because of its positively charged side chain. Thus, the main chain N atom of Gln39, the side chain N atom of Lys58 and the main chain N atom of Gly87 are selected as the atom-level indicators to describe the binding pocket size. Then three distance parameters $d_1$ (the distance between the main chain N atom of Gln39 and the side chain N atom of Lys58), $d_2$ (the distance between the main chain N atom of Gln39 and the main chain N atom of Gly87) and $d_3$ (the distance between the side chain N atom of Lys58 and the main chain N atom of Gly87) are defined to reflect the size of the binding pocket with or without the binding of 2-OG [Fig. 2(C,D)].

**Population shift of binding pocket size upon 2-OG binding**

With the definition of the binding pocket size, it is possible to quantitatively investigate the population shift of the binding pockets in response to the binding of 2-OG. In order to investigate the anticooperativity in the context of distribution shift from unbound to bound PII,

### Table 1

| Energy terms | $P_{II}^{OG1}$ | $P_{II}^{OG2}$ | $P_{II}^{OG3}$ |
|--------------|----------------|----------------|----------------|
| $\Delta G_{ele}$ | -295.3 | -344.0 | -236.4 |
| $\Delta G_{vdw}$ | -2.6 | -2.7 | -2.7 |
| $\Delta G_{MM}$ | -297.9 | -346.7 | -240.6 |
| $\Delta G_{np}$ | -2.7 | -2.7 | -2.7 |
| $\Delta G_{GB}$ | 226.4 | 277.4 | 181.8 |
| $\Delta G_{solv}$ | 223.7 | 274.7 | 179.0 |
| $\Delta G_{binding}$ | -74.1 | -72.0 | -61.6 |

$^a\Delta G_{MM} = \Delta G_{ele} + \Delta G_{vdw}$

$^b\Delta G_{solv} = \Delta G_{np} + \Delta G_{GB}$

$^c\Delta G_{binding} = \Delta G_{MM} + \Delta G_{solv}$

**Figure 2**

Definition of the binding pocket size. (A) Binding free energy decomposed at residue level for PII protein. (B) Hydrogen bonds interactions (dashed lines) between Gln39, Lys58, Gly87, and 2-OG. Definition of binding pocket size by $d_1$, $d_2$ and $d_3$ with (C) or without (D) the binding of 2-OG. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
molecular dynamics simulations of unbound P II trimer (PDB code: 1QY7) were carried out and the distributions of the binding pocket size \(d_1, d_2, d_3\) were calculated (Fig. 3). It is found that although the distances measured from the crystal structure show that they are the same among the monomers (purple dots in Fig. 3), the simulation results show some difference. For instance, \(d_1\) is 10.07 Å and 10.05 Å for chain A and chain B, whereas it is 9.65 Å for chain C. \(d_2\) of chain A (9.85 Å) is somewhat larger than that of chain B (8.10 Å) and chain C (8.17 Å). There are also differences for \(d_3\) among chain A, chain B, and chain C.

Upon binding of the first 2-OG towards chain A of P II trimer, the shift of the binding pocket size of chain B and C is determined and compared with that of chain A. As shown in Figures 3(B) and 4(A), the pocket size of chain B becomes larger as indicated by the distance parameters defined above. In particular, distance \(d_1\) increases from 10.05 Å to 11.47 Å although \(d_2\) and \(d_3\) show little changes. By contrast, the binding pocket of chain C becomes smaller upon the binding of the first 2-OG. This can be seen from the distance \(d_2\), which decreases from 8.17 Å to 6.29 Å and \(d_3\) which decreases from 7.50 Å to 5.62 Å [Figs. 3(C) and 4(B)]. There are only slight changes in terms of \(d_1\).

When the second 2-OG binds to chain B, the binding pocket size of chain C shrinks further because of the decrease of \(d_1\) from 9.43 Å to 7.10 Å and \(d_3\) from 6.29 Å to 4.48 Å [Fig. 4(C,D)]. The distance change of \(d_3\) is very small, only from 5.62 Å to 5.72 Å. The small distance change of \(d_1\) upon the binding of either the first or the second 2-OG may be due to the fact that Lys58 and Gly87 are located in more stable structure areas than Gln39, which resides in the flexible T-loop. The large distance shifts of \(d_1\) and \(d_2\) in response to the ligand binding illustrate the role of P II T-loop in the anticooperative process as well as providing further proof of its role in the ligand binding process.

By defining the binding pocket size with residues that make great contributions to the ligand binding, the anticooperative mechanism of P II protein in response to 2-OG can be well explained as a population shift of the binding pocket size (Fig. 5). Shifts of the binding pocket to become either bigger (in the case of chain B upon the binding of the first 2-OG) or smaller (in the case of chain C upon the binding of the first or the second 2-OG) provide a clear picture of how the P II T-loop interacts with the ligand.
2-OG) will definitely decrease the binding opportunities of 2-OG and result in the negative cooperativity of PII trimer. Results from the ITC measurements suggest that the binding of 2-OG to the first binding site is driven by both enthalpy and entropy ($\Delta H < 0$, $T\Delta S > 0$) with the enthalpy having the bigger impact. The situation is similar for the binding to the second binding site, but here the entropy has the bigger impact. The binding of 2-OG to the third binding site, on the other hand, is clearly driven by the enthalpy ($\Delta H < 0$, $T\Delta S < 0$). The thermodynamic considerations fit well with the binding model illustrated by the change of the binding pocket size. They imply that occupation of the first and second site lead to a more disordered conformation because of the change

**Figure 4**
Distribution of the binding pocket size upon ligand binding. Distance distributions of $d_1$, $d_2$, and $d_3$ for chain B (A) and chain C (B) when the first 2-OG binds towards the binding pocket of chain A as well as distance distributions for chain C (C) when the second 2-OG binds toward the binding pocket of chain B. Purple dots are distances measured based on the crystal structures. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Figure 5**
Sketch of the change of binding pocket size upon the binding of 2-OG. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
of the binding pocket size, whereas occupation of the third site gives a more ordered state because all three sites are now occupied with 2-OG. That is why this reaction is exclusively enthalpy driven.

**Residues mediating the anticooperative signal transduction**

Dynamic correlation analysis [Eq. (4)] was first used to obtain the correlation coefficients between each pair of residues when the first 2-OG binds toward the binding pocket of chain A [Fig. 6(A)] and the second 2-OG toward chain B [Fig. 6(B)]. The number of residues correlated with high correlation coefficient (>0.5) provides a quantitative parameter about the signal-mediating function of each residue [Fig. 6(C,D)]. Then, residues that mediate the signal transduction process with high probability were identified using the algorithm derived from the dynamic correlation analysis with residues identified to illustrate the binding pocket size as $R_s$ or $R_t$ in Eq. (5).

By comparing Figure 6(A,C), we found that the sequence 41 to 50 of chain B shows the largest numbers of correlated residues upon the binding of the first 2-OG. Besides, the sequence 81 to 94 of chain B also show large numbers of correlated residues. Particularly, 21 residues are primarily involved in the signal transduction process from the binding pocket of chain A to that of chain B, of which 7 belong to chain A and 14 belong to chain B [Table II and Fig. 7(A)]. In the case of signaling process from chain A to C, 13 residues contribute to the process, wherein 8 of them belong to chain A and 5 of them belong to chain C [Table II and Fig. 7(B)]. Among these residues, Gln39, Lys40, Ser49, Lys58, Leu59, Gly87, and Asp88 are involved in the signal transduction from chain A to both chain B and C. In the case when the second 2-OG binds towards the binding pocket of chain B [Fig. 6(B,D)], the sequence 43 to 51 of chain C shows the largest numbers of correlated residues. Besides, the sequence 29 to 40 of chain A also shows large numbers of correlated residues. In particular, 14 residues contribute largely to the signal transduction process from chain B to C, of which 6 belong to chain B and 8 belong to chain C [Table II and Fig. 7(C)].

It is not surprising to find that Gln39, Lys58, and Gly87 are involved in each case of ligand binding state due to the fact that they comprise the binding pocket.
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Major Residues Mediating the Signal Transduction from the Binding Site of Chain A to that of Chain B and Chain C upon Binding of the First 2-OG as well as from Chain B to Chain C upon Binding of the Second 2-OG

| Residue no. | Chain A to B | Chain A to C | Chain B to C |
|-------------|--------------|--------------|--------------|
| Phe36ab     |              |              |              |
| Gly37ab     |              |              |              |
| Arg38ab     |              |              |              |
| Glu39ab     |              |              |              |
| Lys40       |              |              |              |
| Gly41ab     |              |              |              |
| Glu42ab     |              |              |              |
| Thr43       |              |              |              |
| Glu44*      |              |              |              |
| Tyr45ab     |              |              |              |
| Arg47ab     |              |              |              |
| Gly48ab     |              |              |              |
| Ser49ab     |              |              |              |
| Leu56       |              |              |              |
| Lys58ab     |              |              |              |
| Leu59ab     |              |              |              |
| Ile66       |              |              |              |
| Gly77ab     |              |              |              |
| Asp88ab     |              |              |              |
| Lys90       |              |              |              |
| Ile91       |              |              |              |
| Phe92       |              |              |              |
| Arg103a     |              |              |              |

The symbol * indicates to which chain the residue belongs to in each case.

*bResidues identified within the time period of 1.0 and 1.5 ns.

Anticooperativity of PII variants by 2-OG

Considering the specificity of Ser49 in the function of PII protein, two variants PII-Ser49Gly and PII-Ser49Asp were constructed and their anticooperativity was measured by isothermal titration calorimetry (ITC) approach. The former is constructed to investigate the role of the side chain in the signal transduction process and the latter is used to mimic the phosphorylation modification of Ser49. As shown in Figure 8, the anticooperativity of PII-Ser49Gly is decreased by approx. 19% upon the binding of the second 2-OG (Kd2/Kd1 from 3.2 to 2.6). The anticooperativity is weakened by approximately 31% upon the binding of the third 2-OG (Kd3/Kd1 from 22.1 to 15.3). In the case where Ser49 is replaced by aspartate acid, the anticooperativity (Kd3/Kd1) is slightly changed from 3.2 to 3.5 upon the binding of the second 2-OG. However, the anticooperativity is strengthened by 19.9% upon the binding of the third 2-OG (Kd3/Kd1 from 22.1 to 26.5) which means PII-Ser49Asp is able to sense a wider range of metabolite concentrations than the wild type if it is expressed in the organism. Moreover, the influence of site mutation on the binding of the third 2-OG is stronger than the second 2-OG as expected by the dynamic correlation analysis.

In order to further confirm the role of T-loop in the anticooperative allosteric regulation of PII, two neighboring sites (Tyr46 and Arg47) that are also located in the mid of the T-loop were chosen and tested. Since these two sites hold large and even positive charged (in the case of Arg47) side chain, both of them were mutated to alanine to decrease the contributions of their side chains and the anticooperativity of the variants were measured using the ITC method. From Figure 8, it can be seen that both variants show the same influence on the anticooperativity upon the binding of the second 2-OG (Kd2/Kd1 from 3.2 to 2.9 and 2.8, respectively). However, Tyr46Ala shows a little stronger influence than Arg47Ala on the anticooperativity upon the binding of the third 2-OG. Tyr46Ala decreases the anticooperativity (Kd3/Kd1) from 22.1 to 19.6 while the anticooperativity is decreases from 22.1 to 20.5 in the case of Arg47Ala. Compared with Ser49, Tyr46 and Arg47 show less influence on the
anticooperativity of PII. This is also expected by the
dynamic correlation analysis. From these experimental
results it can be seen that although the T-loops are long
and extend outward from each subunit, they indeed take
part in the anticooperative allosteric regulation of PII
upon binding of 2-OG. Modifications of residues located
at the T-loop can influence the anticooperativity posi-
tively or negatively.

DISCUSSION

The concept of protein allostery went back to the
MWC model (also known as the concerted model or
symmetry model) put forth by Monod et al. and the
KNF model (also known as the sequential or “induced
fit” model) described by Koshland et al. Both
sought to account for allostery based on gross properties
of the transition between two well-defined end states.
The two models differ most in their assumptions about
subunit interaction and the preexistence of both states.
The concerted model of allostery postulates that subunits
are connected in such a way that a conformational
change in one subunit is necessarily conferred to all
other subunits. Thus, all subunits must exist in the same

Figure 7
Major residues mediating the signal transduction from the binding site of chain A to that of chain B (A) and chain C (B) upon binding of the first
2-OG as well as from chain B to chain C (C) upon binding of the second 2-OG. [Color figure can be viewed in the online issue, which is available
at wileyonlinelibrary.com.]

Figure 8
Anticooperativity of PII variants by 2-OG measured using ITC method.
[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
conformation. The sequential model of allosteric regulation assumes that ligand molecules bind via an induced fit protocol, which converts a subunit from one state to another. However, it does not propagate the conformational change to adjacent subunits. Instead, ligand-binding at one subunit only slightly alters the structure of other subunits so that their binding sites are more receptive or rejective to the ligand. In the case of PII protein, it is known that 2-OG binds in a sequential manner and the binding of 2-OG at one subunit decreases the binding of 2-OG at the other subunits. Thus, it seems that the KNF model is more suitable to describe the anticooperativity of PII by 2-OG. Nevertheless, the underlying mechanism behind the sequential binding of 2-OG is still unclear. Thus it is necessary to get a deeper understanding of the anticooperative allosteric regulation of PII protein in order to reveal the underlying mechanism.

To uncover signal transduction processes within allosteric proteins, several approaches have been developed so far. A co-evolutionary approach, which is based on sequence information of the protein family, was employed to identify amino acid interactions in each protein family. Another novel approach to reveal intramolecular signal transduction networks was proposed based on a new protein dynamics model of energy dissipation. Since correlations in the atomic displacements (dynamic correlation analysis based on fluctuation dynamics) indicate collective motion and are therefore of potential relevance to protein function, correlated motions have been detected in molecular dynamics simulations and used to make deductions concerning dynamical aspects of protein function. In a recent study, molecular dynamics simulation was coupled with co-evolutionary analysis to identify a key residue-residue interaction network which is responsible for the allosteric regulation and signal transduction within allosteric enzymes. In this work, a new approach derived from dynamic correlation analysis is utilized to reveal how the signal is transferred from the binding site of one subunit to the other subunits upon binding of ligand. This approach highlighted amino acid residues mediating the signaling process with high probability. It was surprising to find the important role of PII T-loop in the signal transduction process since the long T-loop extends outward from each subunit and is believed to be responsible for mediating the binding of PII with its target proteins. In particular, it is found that Ser49 also takes part in the allosteric process. The particular impact of this site relies on the fact that it is an important site to regulate the binding of PII towards its target protein N-acetyl-L-glutamate kinase through phosphorylation. Experimental results further confirmed the role of T-loop in influencing the anticooperativity of PII, especially on binding of the third 2-OG. These results not only indicate that the population shift of the binding pocket size is highly correlated to the ligand binding through protein dynamics but also illustrate the versatile function of PII T-loop in the anticooperative allosteric regulation. From the perspective of application, it is possible to alter the sensitivity of PII protein to a much wider range of metabolite concentrations by modifying the residues discovered through the new dynamic correlation analysis approach.

**CONCLUSIONS**

The dynamics of the binding pocket is well characterized by defining its size with residues that contribute greatly to the binding of 2-OG. The anticooperative mechanism of PII protein upon the binding of 2-OG can be well explained by population shift of the binding pocket size. Residues mediating the anticooperative regulation with high probability are discovered by using the new algorithm developed based on dynamic correlation analysis. It is found that Ser49, which is located in the flexible T-loop of PII, plays an important role in the allosteric signaling, as illustrated by experimental results of the anticooperativity of the variants PII-Ser49Gly and PII-Ser49Asp. Overall, the results presented here extend our understanding of the PII T-loop from being essential in versatile binding of target protein to signal-mediating in the anticooperative allosteric regulation.

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REFERENCES

1. Chellamuthu VR, Alva V, Forchhammer K. From cyanobacteria to plants: conservation of PII functions during plastid evolution. Planta 2013;237:451–462.

2. Forchhammer K. P(II) signal transducers: novel functional and structural insights. Trends Microbiol 2008;16:65–72.

3. Espinosa J, Forchhammer K, Burillo S, Contreras A. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate-dependent manner with PII and NtcA. Mol Microbiol 2006;61:457–469.

4. Feria Bourrellier AB, Valot B, Guillot A, Ambard-Bretteville F, Vidal J, Hodges M, Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc Natl Acad Sci USA 2010;107:502–507.

5. Leigh JA, Dodsworth JA. Nitrogen regulation in bacteria and archaea. Annu Rev Microbiol 2007;61:349–377.

6. Bossa C, Anselmi M, Roccatano D, Amadei A, Vallone B, Brunori M, Nolam AD. Extended molecular dynamics simulation of the carbon monoxide migration in sperm whale myoglobin. Biophys J 2004;86:3855–3862.

7. Heinrich A, Maheswaran M, Ruppert U, Forchhammer K. The Synechococcus elongatus PII signal transduction protein controls arginine synthesis by complex formation with N-acetyl-l-glutamate kinase. Mol Microbiol 2004;52:1303–1314.

8. Maheswaran M, Urbanke C, Forchhammer K. Complex formation and catalytic activation by the PII signaling protein of PII signal transduction protein. Proc Natl Acad Sci USA 2010;107:19760–19765.

9. Sant’Anna FH, Trentini DB, de Souto Weber S, Cecagno R, da Silva SC, Schrank IS. The PII superfamily revised: a novel group and evolutionary insights. J Mol Evol 2009;69:517–536.

10. Jiang P, Ninfa AJ, From cyanobacteria to plants: conservation of PII functions during plastid evolution. Planta 2013;237:451–462.

11. Fokina O, Forchhammer K, Burillo S, Contreras A. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate-dependent manner with PII and NtcA. Mol Microbiol 2006;61:457–469.

12. Forchhammer K. P(II) signal transducers: novel functional and structural insights. Trends Microbiol 2008;16:65–72.

13. Espinosa J, Forchhammer K, Burillo S, Contreras A. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate-dependent manner with PII and NtcA. Mol Microbiol 2006;61:457–469.

14. Feria Bourrellier AB, Valot B, Guillot A, Ambard-Bretteville F, Vidal J, Hodges M, Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc Natl Acad Sci USA 2010;107:502–507.

15. Leigh JA, Dodsworth JA. Nitrogen regulation in bacteria and archaea. Annu Rev Microbiol 2007;61:349–377.

16. Bossa C, Anselmi M, Roccatano D, Amadei A, Vallone B, Brunori M, Nolam AD. Extended molecular dynamics simulation of the carbon monoxide migration in sperm whale myoglobin. Biophys J 2004;86:3855–3862.

17. Heinrich A, Maheswaran M, Ruppert U, Forchhammer K. The Synechococcus elongatus PII signal transduction protein controls arginine synthesis by complex formation with N-acetyl-l-glutamate kinase. Mol Microbiol 2004;52:1303–1314.

18. Maheswaran M, Urbanke C, Forchhammer K. Complex formation and catalytic activation by the PII signaling protein of N-acetyl-l-glutamate kinase from Synechococcus elongatus strain PCC 7942. J Biol Chem 2004;279:55202–55210.

19. Sant’Anna FH, Trentini DB, de Souto Weber S, Cecagno R, da Silva SC, Schrank IS. The PII superfamily revised: a novel group and evolutionary insights. J Mol Evol 2009;69:517–536.

20. Jiang P, Ninfa AJ. Escherichia coli PII signal transduction protein to regulate the activ-
42. Gunasekaran K, Ma B, Nussinov R. Is allostery an intrinsic property of all dynamic proteins? Proteins 2004;57:433–443.
43. Kumar S, Ma B, Tsai CJ, Sinha N, Nussinov R. Folding and binding cascades: dynamic landscapes and population shifts. Protein Sci 2000;9:10–19.
44. Luque I, Leavitt SA, Freire E. The linkage between protein folding and functional cooperativity: two sides of the same coin? Annu Rev Biophys Biomol Struct 2002;31:235–256.
45. Lockless SW, Ranganathan R. Evolutionarily conserved pathways of energetic connectivity in protein families. Science 1999;286:295–299.
46. Suel GM, Lockless SW, Wall MA, Ranganathan R. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. Nat Struct Biol 2003;10:59–69.
47. Ma CW, Xiu ZL, Zeng AP. A new concept to reveal protein dynamics based on energy dissipation. PLoS One 2011;6:e26453.
48. Ma CW, Xiu ZL, Zeng AP. Discovery of intramolecular signal transduction network based on a new protein dynamics model of energy dissipation. PLoS One 2012;7:e31529.
49. Burillo S, Luque I, Fuentes I, Contreras A. Interactions between the nitrogen signal transduction protein PII and N-acetyl glutamate kinase in organisms that perform oxygenic photosynthesis. J Bacteriol 2004;186:3346–3354.
50. Daniel RM, Dunn RV, Finney JL, Smith JC. The role of dynamics in enzyme activity. Annu Rev Biophys Biomol Struct 2003;32:69–92.
51. Garcia AE, Hummer G. Conformational dynamics of cytochrome c: correlation to hydrogen exchange. Proteins 1999;36:175–191.
52. Showalter SA, Hall KB. A functional role for correlated motion in the N-terminal RNA-binding domain of human U1A protein. J Mol Biol 2002;322:533–542.
53. Keskin O, Durell SR, Bahar I, Jernigan RL, Covell DG. Relating molecular flexibility to function: a case study of tubulin. Biophys J 2002;83:663–680.
54. Ertekin A, Nussinov R, Haliloglu T. Association of putative concave protein-binding sites with the fluctuation behavior of residues. Protein Sci 2006;15:2265–2277.
55. Chen Z, Rappert S, Sun JB, Zeng AP. Integrating molecular dynamics and co-evolutionary analysis for target prediction and engineering of allosteric regulation of aspartokinases for amino acids production. J Biotechnol 2011;154:248–254.
56. Chen Z, Meyer WQ, Rappert S, Sun JB, Zeng AP. Coevolutionary analysis enables rational deregulation of allosteric enzyme inhibition in Corynebacterium glutamicum for lysine production. Appl Environ Microbiol 2011;77:4352–4360.
57. Zeth K, Fokina O, Forchhammer K. An engineered PII protein variant that senses a novel ligand: atomic resolution structure of the complex with citrate. Acta Crystallogr D: Biol Crystallogr 2012;68:901–908.