Beyond static structure: X-ray solution scattering: MAXS reveals a massive movement during catalytic action of the non-phosphorylated human kinase MAP2K4

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Abstract

Small angle X-ray scattering (SAXS) is a well-known technique for analyzing the size and shape of proteins in solution. Standard SAXS uses data below about q = 0.25 Å^{-1}. Therefore, SAXS can only provide information regarding size changes in the target molecule, aggregation, and approximate molecular shape. On the other hand, X-ray scattering in the middle-angle region (q = 0.30–0.75 Å^{-1}) contains important information for analyzing molecular structure and conformational changes in solution, such as the distance between intramolecular tertiary structures and the distance between secondary structures. By using the data in this middle-angle region, we can visualize more detailed molecular behavior and conformational changes. The solution scattering method that includes this important middle-angle region information is named "middle angle X-ray scattering (MAXS)". In this article, we introduce the "massive movement" of the structure of human kinase MAP2K4 in solution, which was revealed by structural ensemble analysis using MAXS.

1. Introduction

Proteins in solution change conformation locally or globally, and this flexibility is closely related to the expression of function. Understanding this conformational change is a necessary condition for understanding protein function and, by extension, biochemical phenomena. Single-crystal structural analysis by diffraction and single-particle analysis using cryo-electron microscopy are two of the methods used to obtain protein structural information. While these methods can provide high-resolution structural information, they cannot provide the structure of the protein under physiologically relevant conditions in solution because the molecular structure may be distorted by crystal packing forces or under cryo-cooled conditions.

On the other hand, X-ray solution scattering is one of the few methods that can analyze the native protein structure unaffected by crystal packing or cryo-cooling, because it does not require either technique. Furthermore, the sample is irradiated with X-ray for measurement and analysis in solution. In particular, the middle-angle region (q = 0.30–0.75 Å^{-1}) of X-ray solution scattering, which contains information, such as the distance between intramolecular domains and the distance between secondary structures, can be used for analysis to visualize more detailed molecular behavior and conformational changes[1]. By using solution scattering in the middle-angle X-ray scattering (MAXS) regime, which includes distance information in this important middle-angle region, it is possible to capture the conformational changes of proteins in solution, including flexible conformational changes.

What kind of conformational changes can we visualize? The results of the analysis of "movement" in solution will be presented using human-derived MAP2K4. MAP2K4 is a serine/threonine kinase that phosphorylates and activates JNK and P38 MAPKs, which are involved in the regulation of cell differentiation, apoptosis, inflammation and other biological functions in response to environmental stress. MAP2K4 is involved in carcinogenesis and has been reported to promote carcinogenesis in skin cancer[2]. Therefore, MAP2K4 has attracted attention as a target for cancer therapy.

In this article, we introduce the solution structure analysis of three different states (apo form, a two-component complex with AMP-PNP, a non-hydrolyzable analog of ATP, a binary complex, and a three-component complex with AMP-PNP and p38α peptide, a substrate, a ternary complex) of the kinase domain of human non-phosphorylated MAP2K4 (hereafter abbreviated as MAP2K4), which is much more flexible in solution than the crystal structure would suggest. The putative mechanisms of MAP2K4 functional regulation and new drug discovery strategies will be presented based on the knowledge of this movement.

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2. Experiment

2.1. Non-phosphorylated human MAP2K4

Human MAP2K4 expressed in E. coli was used as a validation sample for conformational change analysis of flexible proteins[3]. Apo, binary and ternary complexes were prepared according to Matsumoto et al.[3]–[5].

2.2. MAXS measurements and analysis

All measurements in this experiment were performed with a BioSAXS equipped with an ultra-high-intensity microfocus rotating anode X-ray generator FR-X as the X-ray source and a photon-counting X-ray detector as the detector. Each scattering image was radially averaged using SAXSLab, the BioSAXS control and processing software, to obtain one-dimensional scattering intensities. Subsequent data processing and analysis were performed using the ATSAS program package[6], and figures were created in PyMol[7].

3. Dynamic structural analysis of MAP2K4

3.1. Single crystal structure analysis

MAP2K4 has a typical kinase domain consisting of two lobes, the N-terminal lobe and the C-terminal lobe, connected by a hinge region. This hinge region consists of a single flexible loop structure, which is responsible for the overall structural flexibility of kinases, including MAP2K4. The crystal structures of the apo, binary, and ternary complexes of MAP2K4 are shown in Fig. 1, and superposition of the apo and binary complexes or the binary and ternary complexes are shown in Fig. 2[3], [4]. The characteristics of each structure are described below.

Apo form (PDB code 3vut): two molecules were present in the asymmetric unit. The molecule on the left (light blue) could not be observed in the crystal structure due to structural disorder with large conformational changes in the N-lobe except for the αC helix.

The molecule on the right (magenta) displayed the complete structure including the N-lobe as a result of suppression of the conformational change of the N-lobe due to crystal packing.

The N-lobe formed a "pseudo β-barrel like structure," a structural motif not seen in other typical kinases.

Therefore, it is clear that the pseudo β-barrel like structure is a conformational change in the left molecule, but the conformational change direction (left, right, up, down, diagonal, etc.) cannot be speculated (Fig. 1).

Furthermore, superposition with the binary complex in Fig. 2 suggests that the apo form is elongated (dashed green circle) and appears to expose the ATP-binding site.

Binary complex (PDB code 3aln): three molecules were located in the asymmetric unit. Two of the molecules (blue and red) formed a domain-swapped dimer in which the N-terminus of each molecule inserted into each other's N-lobe. As shown in Fig. 2, they formed a compact structure that enclosed the AMP-PNP.

Ternary complex (PDB code 3alo): only one molecule was present in the asymmetric unit. The superposition of the binary-ternary complexes in Fig. 2 shows that the activation loop transforms from a coil to a helical activation loop, a long α-helix, which blocks the access of substrates and inhibitors to the ATP-binding pocket, resulting in the construction of a robust auto-inhibitory structure.

While the crystal structure revealed many things, it also raised several new questions.

- The functional unit of MAP2K4 could not be determined because the number of molecules in the asymmetric unit is different in all three states of the

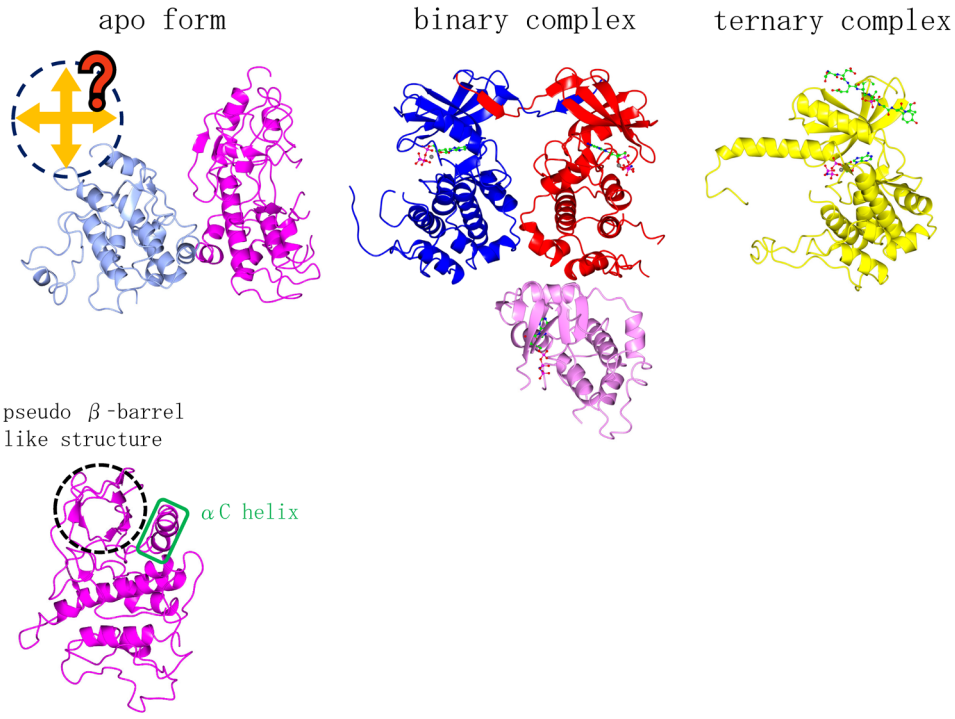

Fig. 1. Crystal structures of apo form, binary complex and ternary complex.

crystal structure.

- Although the Apo form was elongated, it is possible that the N and C-lobe are connected by a flexible hinge region, resulting in an elongated and deformed structure due to crystal packing.
- What is the direction of conformational change of the pseudo-β-barrel-like structure of the Apo form?
- In the Apo form, most of the activation loop located in the neighborhood of the ATP-binding pocket was disordered. Therefore, it is possible that the actual ATP-binding pocket is covered by the activation loop and not exposed.
- Does the dimer formed by domain swapping in the binary complex still exist in solution?
- Since crystal structures are often affected by crystal packing and freezing, would each of the three states be a structure affected by them?

To address these questions, we decided to perform a solution structure analysis of each state using MAXS.

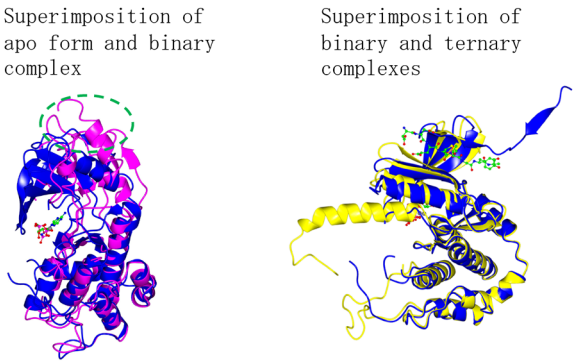

Fig. 2. Superimposed view of crystal structures.

3.2. Solution structure analysis using bead modeling method

First, the molecular weight was estimated using the forward scattering intensity $I(0)$ calculated by Guinier analysis using the experimental scattering intensities of the apo form, binary and ternary complexes and the protein concentration, indicating that the MAP2K4 was present in solution as a monomer in all states. The number of molecules in the asymmetric unit of the crystal did not reflect the state in solution.

Next, the experimental scattering intensity for each state was superimposed on the theoretical scattering intensity for one molecule of each crystal structure calculated by CRYSOL[8] (Fig. 3a). The separation and χ^2 values of the theoretical and experimental scattering intensity graphs for all states indicate that there is a clear difference between the structure in solution and crystal[5]. The crystal structure is a static structure affected by crystal packing, indicating that it does not fully reflect the behavior of the protein in solution. This reveals the importance of solution structure analysis using MAXS. Therefore, we performed solution structure analysis using GASBOR, an *ab initio* bead modeling method[9]. Figure 4 shows a scattering intensity plot for each state and the crystal structure superimposed on the calculated bead model. This superimposition suggests that the apo form is more elongated in solution than the crystal structure. The combination of this elongated solution structure and the disordered crystal structure suggests that the pseudo-β-barrel structure is likely to fluctuate up and down. The bead model of the apo form and the binary complex indicates that MAP2K4 undergoes a morphological change to a compact structure upon binding of AMP-PNP. In the ternary complex, the bead

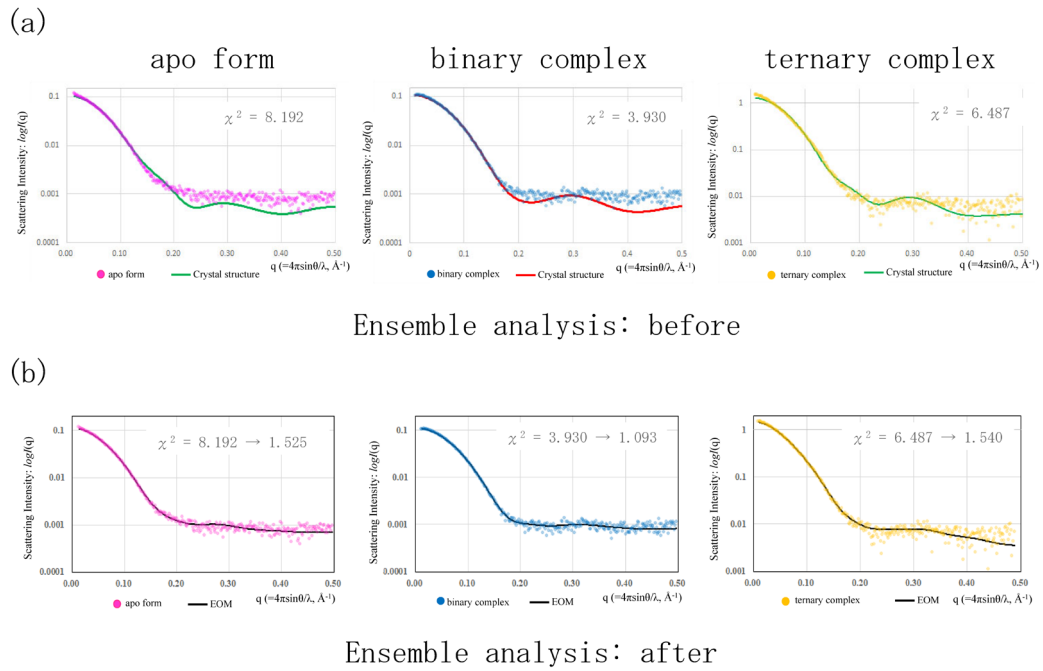

Fig. 3. Superimposition of experimental scattering intensity and theoretical scattering intensity plots. (a) Before structural ensemble analysis. (b) After structural ensemble analysis.

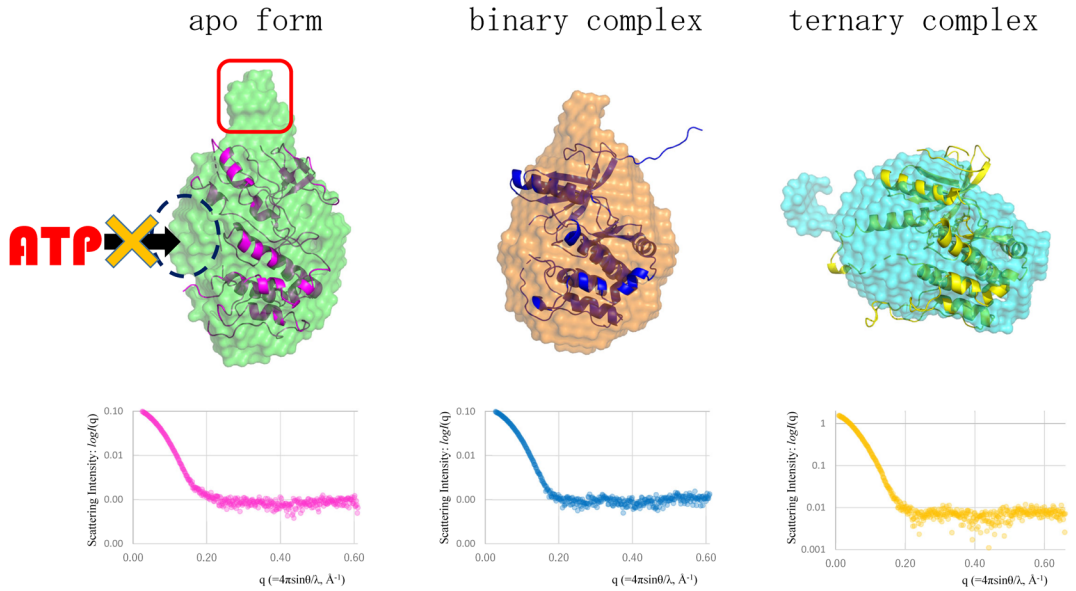

Fig. 4. Superimposed views of the bead models and crystal structures of the apo form, binary complex, and ternary complex.

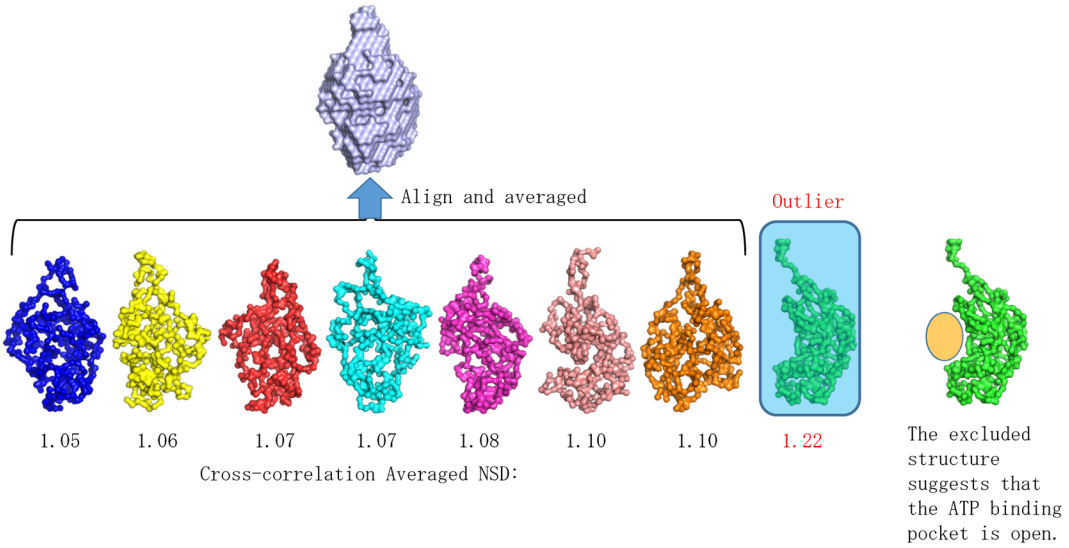

Fig. 5. Problems with the bead modeling method.

model had a horizontally spread-out shape, showing the influence of the morphological change to a long α-helix. However, throughout all of these superpositions, the agreement between the solution and crystal structures was not high, indicating that the crystal structures were affected by crystal packing and did not necessarily reflect the functional state in solution.

By performing the bead modeling method, it can be said that we were able to visualize the structure of MAP2K4 in each state, free from the effect of crystal packing. On the other hand, the following new questions were raised.

- The bead model of the apo form suggests that the ATP-binding pocket indicated by the dashed box is covered by a structure of the protein and not exposed to the solvent side. This makes it difficult to understand how MP2K4 binds to ATP, since the ATP-binding pocket is not exposed to the solvent side.
- In all three states, there are regions that do not match for the superposition of the solution and crystal structures.
- Proteins are known to be flexible, but it may be impossible to represent this flexible structure in a single bead model.

Therefore, the procedure of the bead modeling method was discussed.

3.3. Limitations of the bead modeling method

In the bead modeling method, the bead model is constructed multiple times, multiple bead models are aligned, the models are compared, and the averaged structure is adopted as the final solution structure, excluding models that are far from other models. Figure 5 shows the calculation process for the actual apo form bead model. In this calculation, eight bead models were constructed and compared with each other,

and the average structure was obtained using only the models with high agreement. The structure surrounded by the blue box on the far right is the one that was excluded from the average structure calculation because it was far from the other models. A careful look at this excluded structure may indicate that MAP2K4 in the apo form may be more vertically elongated and may also have a structure that exposes the ATP binding pocket toward the solvent. In a highly flexible protein, is it not conceivable that structures that have a significant bearing on the function of the protein are hidden in the structures that are eliminated in the averaging process? By eliminating such possible structures, we may have a wrong understanding of the protein's function in solution. Based on these considerations, we came to the conclusion that it was necessary to analyze proteins in solution in consideration of the fact that they have multiple different states. Therefore, we attempted the use of the ensemble optimization method (EOM [10]) of ATSAS, in which proteins in solution are analyzed as a structural ensemble with multiple states.

3.4. Structural ensemble analysis with EOM

The EOM generates a population of about 10,000 various state models based on PDB coordinate information as initial structure states, and calculates the theoretical scattering intensity of each state model. Then, multiple model structures are selected and compared to match the experimental scattering intensity data for further optimization (Fig. 6). The EOM was used to perform a structural ensemble analysis of the three states of MAP2K4. The monomeric structure of each crystal structure was used for the initial structural state. The resulting superposition of scattering curves is shown in Fig. 3b. The χ^2 values were close to unity in all states, suggesting that each state of MAP2K4 is dynamic in solution and moves between the structural ensembles obtained by EOM. As a result, questions about the process of ATP binding that had not been resolved by the crystal structure and bead modeling methods were addressed as new findings by the MAXS + EOM analysis. Because almost all proteins may have conformational changes, there is a need to perform structural ensemble analysis using MAXS. The results of EOM analysis of the apo form, binary complex, and ternary complex are described below.

3.4.1. Structural ensemble model: apo form

First, let us introduce the apo form. The structural ensemble analysis resulted in four representative structures for the apo form, as shown in Fig. 7. The numbers in parentheses under each representative structure indicate the relative ratio.

Apo-1, 2, and 3 form compact conformations, which are relatively similar to the crystal structure, but the ATP-binding pocket is covered by the N-lobe and activation loop, forming a structure that is inaccessible to ATP despite being an apo form. In contrast, apo-4, with a low relative ratio of 11%, forms an elongated conformation with a pseudo-β-barrel structure with a large upward rotation. As a result, the ATP-binding pocket is significantly exposed to the solvent, allowing ATP to smoothly access the pocket.

3.4.2. Structural ensemble model: binary complex

Next, we describe the results of the structural ensemble analysis of the binary complex: the binary complex also had four representative structures, with binary-1 and 4 being similar (Fig. 8a). Before the analysis, we expected that the structure itself would not differ significantly from the crystal structure, even if the N-terminal domain had some fluctuations, because the binary complex has a compact structure bound to ATP. However, this expectation was overturned when the representative structures were superimposed on the crystal structure (Fig. 8b). In solution, the N-lobe of

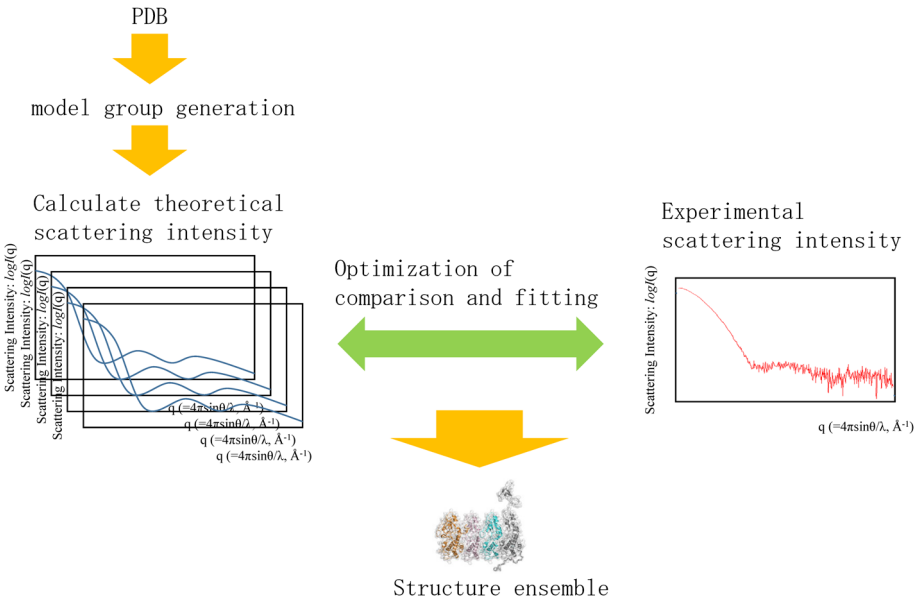

Fig. 6. Schematic diagram of the structural ensemble method.

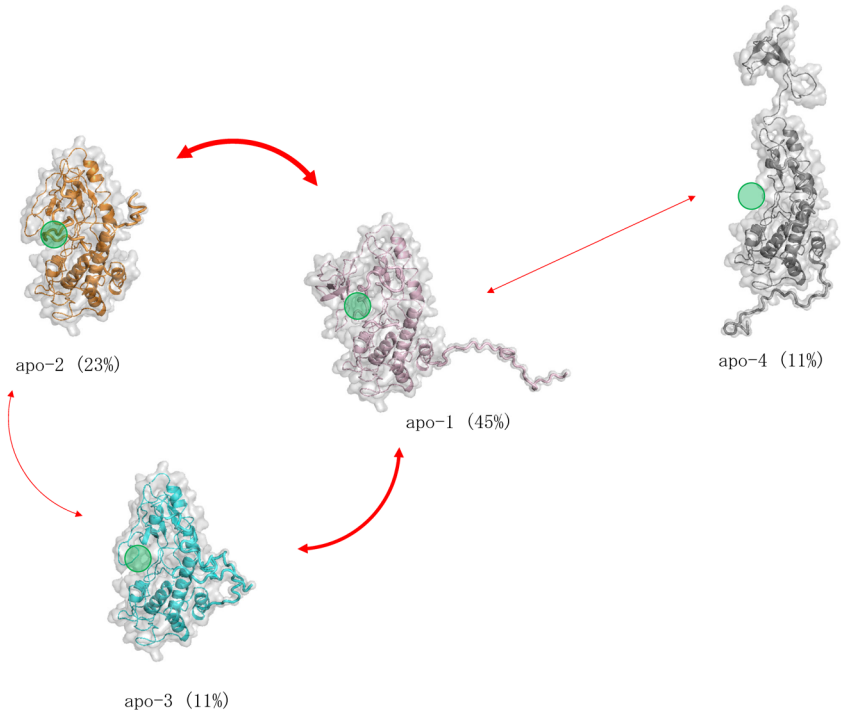

Fig. 7. Dynamic structure by structural ensemble analysis of the apo form.

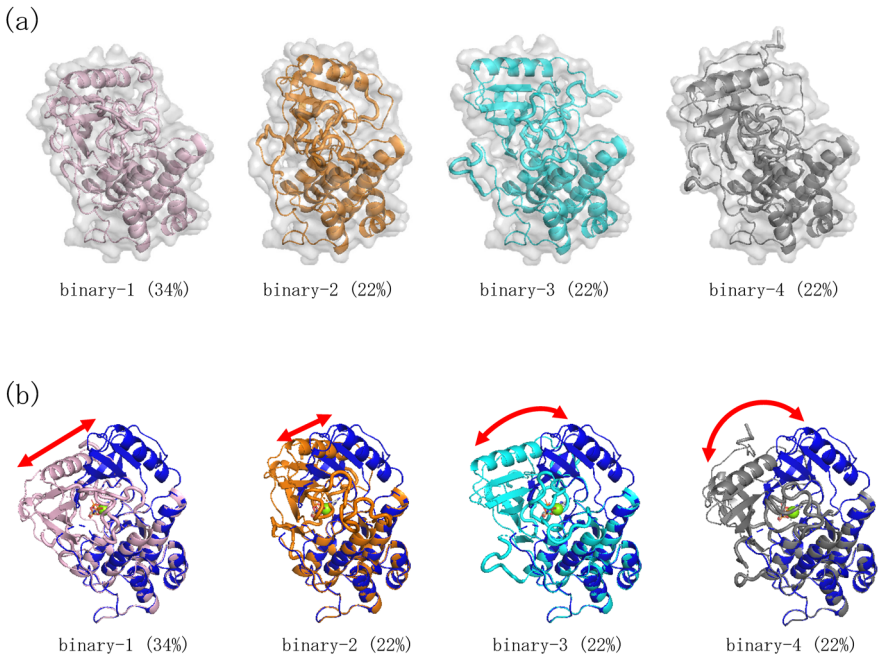

Fig. 8. Dynamic structure by structural ensemble analysis of the binary complex.

the binary complex was more inclined toward the ATP binding pocket than the crystal structure, and ATP was correspondingly wrapped around the N-lobe and C-lobe, and bound more tightly. As a result, the binary complex was even more compact than the crystal structure. This is thought to regulate the non-phosphorylated MAP2K4 so that it does not accidentally phosphorylate downstream kinases. In this way, MAP2K4 may achieve strict on/off control of signaling in vivo.

3.4.3. Structural ensemble model: ternary complex

Finally, a structural ensemble analysis of the ternary complex was performed. The ternary complex had three representative structures (Fig. 9a). The three representative structures differed significantly from each other. Ternary-1 formed a compact structure, while ternary-3 formed a structure in which the N-lobe was far away from the helical activation loop, as if it had a widely opened mouth. Ternary-2 formed a structure in which the N-lobe was far from the helical activation loop, although the distance was not as far as that of ternary-3, and was considered to be an intermediate state between ternary-1 and 3.

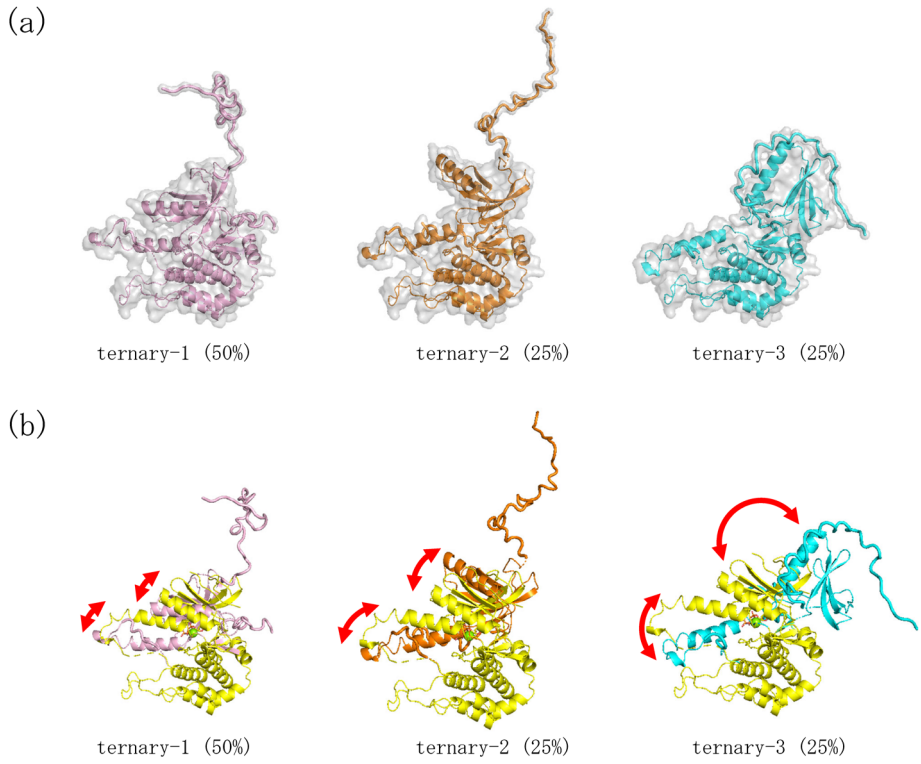

Fig. 9. Dynamic structure by structural ensemble analysis of the ternary complex.

From the superposition diagram with the crystal structure in Fig. 9b, ternary-1 has the highest relative ratio and forms the most similar structure to the crystal structure. However, the N-lobe and helical activation loop are rotated toward the ATP binding pocket by about 21° compared to the crystal structure, suggesting that they are bound to wrap around ATP as in the binary complex. In ternary-2, the N-lobe and helical activation loop were found to be rotated while tilted away from each other. As a result, a V-shaped depression appeared between the N-lobe and helical activation loop. Surprisingly, in ternary-3, the N-lobe and the helical activation loop rotated further apart, forming a horizontally elongated structure with the N-lobe and C-lobe wide open. Comparison with the crystal structure revealed that the ternary complex undergoes a surprising structural change centered on the N-lobe, which is not seen elsewhere.

In ternary-2 and 3 in Fig. 9, the N-lobe is rotated backward, and at first glance, it seems that substrate kinase can access ATP. In reality, however, the substrate kinase cannot access ATP due to steric hindrance by the helical activation loop. As a result, the auto inhibitory structure is maintained. Thus, the long helix structure of the activation loop, which is maintained in both representative structures, is a prerequisite for the auto inhibition of non-phosphorylated MAP2K4.

In non-phosphorylated MAP2K4, the substrate kinase binds to allosteric sites in the N-lobe, resulting in a major conformational change of the N-lobe. This N-lobe flexibility may lead to partial destabilization of MAP2K4 leading to aggregation or accelerated enzymatic reaction.

3.5. Effect of crystal packing

Why did the crystal structure and solution structure differ so greatly? One possible explanation is the effect of crystal packing. Crystal packing minimizes protein movement, resulting in good quality crystals that can be analyzed in detail by high-resolution structural analysis. On the other hand, the crystal structure is a stationary structure trapped in the crystal packing, so it is impossible to see the large movement of the protein. Sometimes, the shape of the molecule is deformed by the crystal packing. Thus, advantages and disadvantages coexist in crystal packing.

The packing of MAP2K4 presented in this article is shown in Fig. 10. The neighboring molecules suppress the fluctuations, rotations, and vibrations of the N-lobe of MAP2K4 in each state. From this figure, it can be seen that the crystal structure is still a static structure, and solution structure analysis by MAXS is essential to obtain structural information on a dynamic protein in motion. In addition, when there are flexible and large conformational changes, as in the case of MAP2K4, detection of a conformation with a low population is only possible by solution scattering analysis.

3.6. Stability of MAP2K4 and the effect of p38α peptide binding on MAP2K4 activity.

The results of the structural ensemble analysis suggested that the apo state has a pseudo-β-barrel structure with upward rotation, the binary complex has a rigid structure, and the ternary complex has a fairly

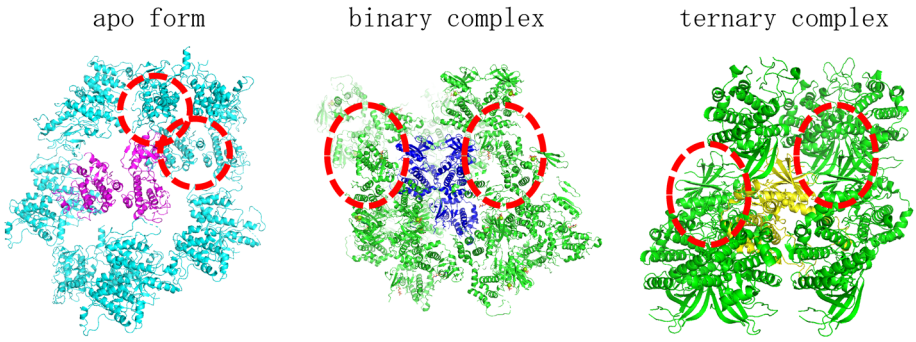

Fig. 10. Effect of crystal packing on the apo form, the binary complex and the ternary complex.

flexible structure, suggesting that the three states have three different conformational characteristics. To further understand the regulatory mechanism of MAP2K4, we evaluated the molecular stability of each state using Differential Scanning Calorimetry (DSC) and measured the cascade kinase activity.

3.6.1. Differential Scanning Calorimetry: DSC

Differential scanning calorimetry (DSC) results showed that the Tm values of the binary and ternary complexes were significantly higher than those of the apo form by 9°C and 10°C, respectively, indicating increased stability (Fig. 11a). This suggests that ATP binding is key to the structural stabilization of MAP2K4.

The ternary complex is more stable than the ATP-only complex when bound to the substrate kinase. On the other hand, the crystal structure of the binary complex suggested the possibility of dimerization by domain swapping. However, the MAXS results indicate that the binary complex is monomeric in solution. From this result, the dissociation constant of the binary complex is estimated to be large (Fig. 11b). During the Covid-19 pandemic, people maintained social distance from each other to prevent the spread of infectious disease. Similarly, MAP2K4 maintains social distance under normal conditions, but in close crystal-like proximity to each other, it is thought to indicate that MAP2K4 becomes a dimer like an arms around each other's shoulders.

However, why does the ternary complex have only one degree increase in Tm value compared to the binary complex despite the binding of p38α peptide, the original substrate kinase? The results of structural ensemble analysis indicate that the ternary complex is a flexible structure with a large N-lobe, especially around the phosphorylation site. Taken together, the results of DSC and structural ensemble analysis suggest that the increased stability due to p38α peptide binding may be offset by destabilization caused by the increased flexibility of the N-lobe (Fig. 11c).

3.6.2. Cascade kinase activity assay (ELISA)

Next, cascade kinase activity assays were performed to determine whether the substrate kinase, p38, promotes, inhibits, or has no particular effect on MAP2K4 activity. The results showed that p38α peptide inhibits MAP2K4 activity in a concentration-dependent

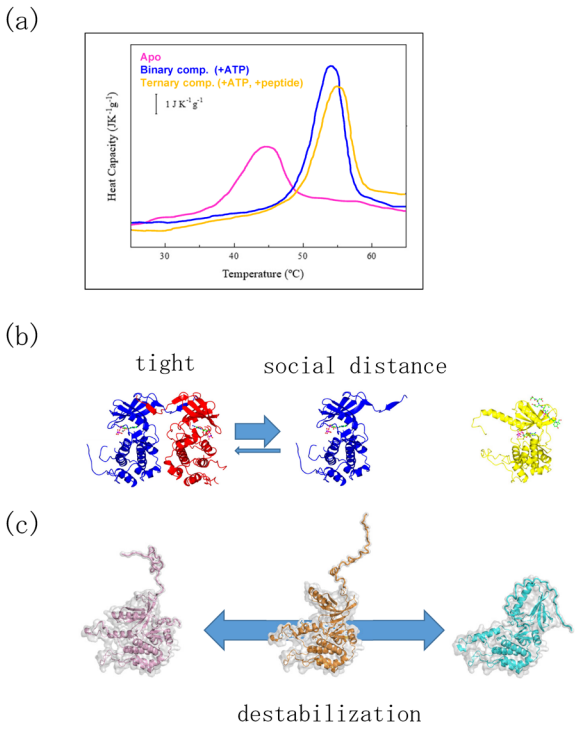

Fig. 11. Differential scanning calorimetry.

manner. Taken together with the results of the structural ensemble analysis, it is possible that p38α peptide accelerates the conformational fluctuations of MAP2K4, making MAP2K4 more susceptible to degeneration.

3.7. Newly led non-phosphorylated MAP2K4 regulatory mechanism

We describe a novel and detailed non-phosphorylated MAP2K4 regulatory mechanism derived from crystal structure analysis, solution scattering analysis, structural ensemble analysis, DSC, and cascade kinase activity assay results (Fig. 12).

3.7.1. Apo form

In the apo form, the structure of MAP2K4 was found to be highly variable in solution. This result suggests the following regulatory mechanism in the apo form (Fig. 12a).

- Normally, the ATP binding pocket is in a compact apo close conformation covered with Gly rich loop and activation loop, and the conformational change occurs

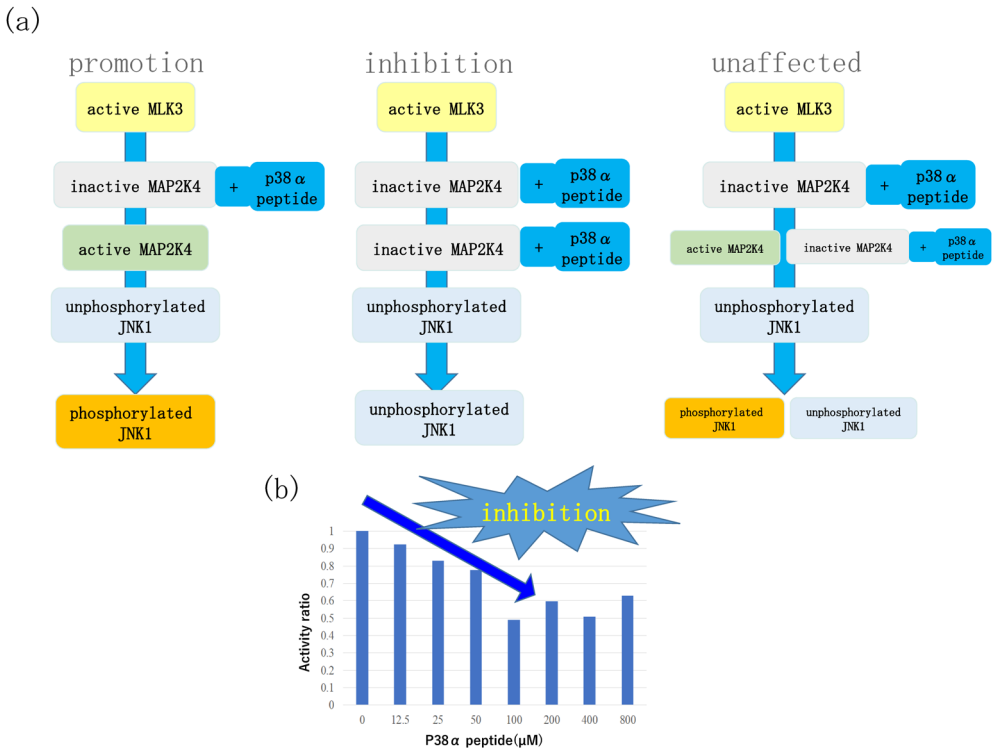

Fig. 12. Cascade kinase activity assay.

when the N-lobe oscillates from side to side.

- The pseudo β-barrel structure is rotated upward by a relative ratio of approximately 10% to form an extended apo open conformation in which the ATP binding pocket is exposed to solvent and ATP is accessible.
- When the apo form transitions from the open to the closed conformation, the approaching ATP is held in the ATP-binding pocket.
- Transition to the binary complex conformation.

Therefore, it can be said that the apo form is waiting for ATP to approach it with a large conformational change in solution. Under normal conditions where ATP is abundant, even apo-4 with a low relative ratio of 10% has a good chance to bind ATP. However, let us consider a severe situation in which ATP is extremely low in the cell. In this situation, with a low relative ratio of apo-4 that can bind to ATP and only a small amount of ATP present, the chances of the apo form being able to bind to ATP are expected to be quite low. This may act as a kind of defense mechanism to ensure that ATP can be preferentially used as fuel for vital activities such as cell maintenance and transport in critical situations. In the apo form, the hydrophobic amino acids of the αC helix are exposed to the solvent side, which can be imagined to be a situation that promotes aggregation. Therefore, it is possible that the mechanism is that when the apo form is kept in the apo state for a long time, aggregates are formed based on the exposed hydrophobic amino acids and then eliminated as aggregates.

3.7.2. Binary complex

In the presence of ATP, MAP2K4 binds to ATP more tightly than expected from its crystal structure, forming the most compact structure of the three states. In the binary complex, the activation loop is always pushed toward the solvent side, which means that the activation loop is easily phosphorylated (Fig. 12b). On the other hand, in the non-phosphorylated MAP2K4, the γ-phosphate of ATP, which is the original substrate kinase binding site, is covered by the activation loop that is pushed toward the solvent side, and the substrate kinases, JNK and p38, cannot access ATP.

This firmly prevents signaling errors due to misphosphorylation.

3.7.3. Ternary complex

In non-phosphorylated MAP2K4, substrate kinase bound at the allosteric region rather than at the γ-phosphate site of ATP. This binding resulted in a morphological change of the activation loop from a coil to a helical activation loop, a long α-helix, and a significant increase in the flexibility of the N-lobe. The N-lobe is bent backward, exposing the phosphorylation site on the helical activation loop, which allows phosphorylation by the upstream kinase (Fig. 12c). On the other hand, substrate kinases cannot approach ATP due to the steric hindrance caused by the helical activation loop, thus maintaining the autoinhibitory structure and preventing signal transduction errors here as well.

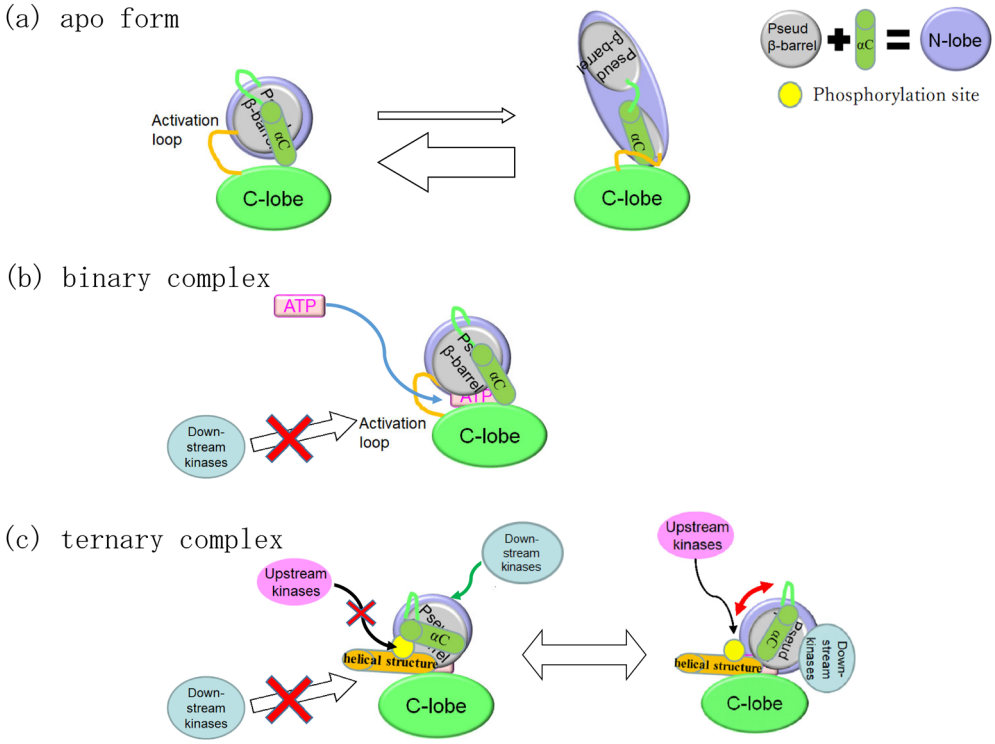

Fig. 13. A novel, detailed regulatory mechanism for non-phosphorylated MAP2K4.

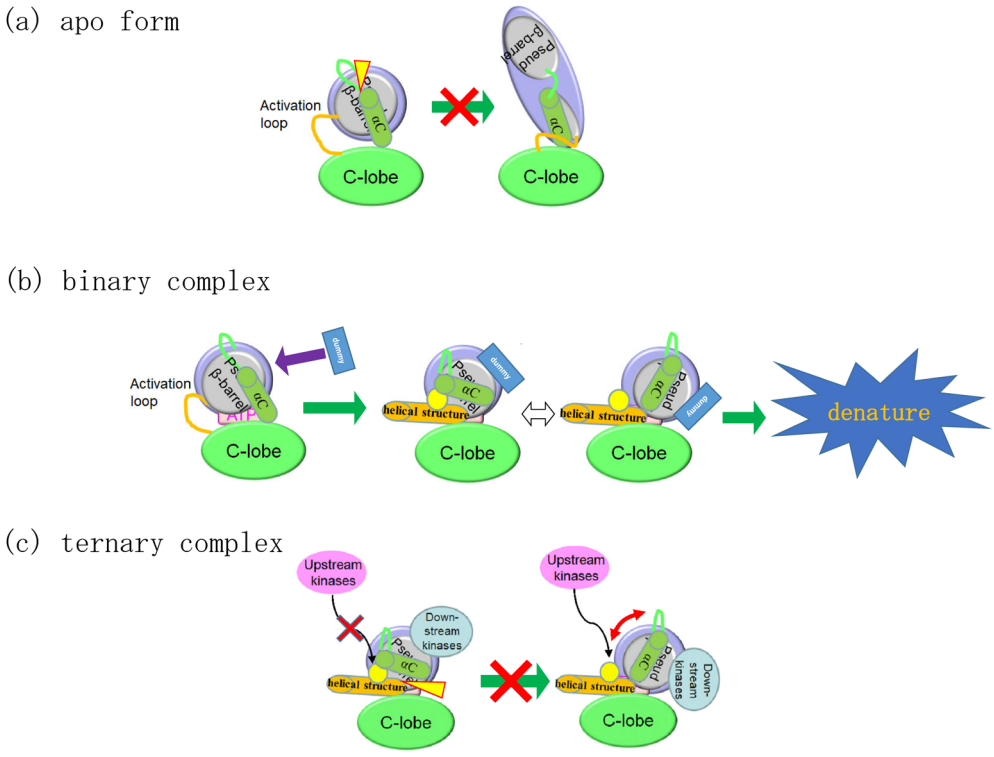

Fig. 14 New drug discovery strategies based on "protein dynamics".

3.8. New drug discovery strategies based on "protein dynamics" is named "dynamics employed structure based drug design: DESBDD"

Thus, a new regulatory mechanism of MAP2K4 was proposed. We believe that focusing on the "dynamics" of MAP2K4 based on this new regulatory mechanism may lead to a new approach to drug discovery (Fig. 14).

3.8.1. Apo form

Structural ensemble analysis shows that the pseudo-β-barrel structure of the apo form rotates upward, thereby exposing the ATP binding pocket and allowing

access to ATP. If the pseudo-β-barrel structure cannot rotate upward, MAP2K4 cannot bind ATP, and the non-phosphorylated MAP2K4 is preserved, and substrate kinase phosphorylation is inhibited. Therefore, if we can design a wedge-shaped compound between the pseudo-β-barrel structure and the αC helix to inhibit the upward rotation of the pseudo-β-barrel structure, we may be able to pinpoint and inhibit MAP2K4 (Fig. 14a).

3.8.2. Binary complex

Next, let us consider the binary complex. The crystal structure of non-phosphorylated MAP2K4 shows that the substrate peptide binds to the allosteric site. Structural ensemble analysis and DSC revealed that the binding of the substrate peptide to the allosteric site increases the flexibility of the N-lobe, resulting in the destabilization of MAP2K4. What would happen if a peptide with high substrate peptide-like binding affinity is bound to the allosteric site? This would force a pseudo-ternary complex, which would result in the destabilization of MAP2K4 by structural fluctuation and ultimately destabilize, denature, and eliminate only MAP2K4 (Fig. 14b). Such a fluctuation-accelerated drug discovery is also possible.

3.8.3. Ternary complex

Finally, let us consider the ternary complex. In the ternary complex, the flexibility of the N-lobe is increased, and the phosphorylation site on the helical activation loop is exposed when the N-lobe turns over. In this state, it is thought that MAP2K4 can be shifted to the active form by approaching the upstream kinase. However, by placing a wedge-shaped compound between the helical activation loop and the αC helix from behind the N-lobe, the phosphorylation site on the helical activation loop would not be exposed. As a result, MAP2K4 would not be able to transition to the active form, and MAP2K4 would be maintained in the inactive form, resulting in a pinpoint inhibitor of MAP2K4 (Fig. 14c). Contrary to the previous point, fluctuation-inhibitory drug discovery is another possible approach.

A drug discovery strategy that goes beyond traditional structure-based drug design (SBDD) and takes into account the "protein dynamics" is called dynamics employed structure-based drug design (DESBDD).

4. Summary

It is difficult to understand the complicated mechanism of functional regulation of a highly flexible protein such as the non-phosphorylated MAP2K4 described in this article only from its crystal structure. This is because the crystal structure is a static structure affected by crystal packing forces, and thus, only one aspect of functional regulation can be observed. Therefore, it is important to add "dynamic" structural information in order to understand the complicated function regulation mechanism. Therefore, for future structural analysis of proteins and elucidation of their functional regulatory mechanisms, it is essential to combine with analytical methods that can provide "dynamic" information. The MAXS experiment is one of the few methods that can provide structural information on the "dynamic" conformational changes of proteins in solution and we believe to be one of the most effective methods to combine with other methods. As described in this article, by combining MAXS analysis, it has become possible to obtain dynamic "living state" information. By using this information, we can gain access to more detailed new knowledge, solve problems that have hindered our understanding, and potentially reveal new structure-function relationships.

In addition, as introduced in the last section, the use of "protein dynamics," which has not been focused on until now, may enable the development of "novel molecularly targeted drugs" that pinpoint target proteins in a different way from the conventional approach. By the way, the analysis of "dynamics" described in this paper is based on crystal structures. Therefore, it is undeniable that some researchers feel that it is a difficult task. However, it is now possible to use predicted structures such as AlphaFold2[(11)] as initial structure states for structural ensemble analysis. Therefore, the "dynamics" analysis using MAXS is not as difficult as one might think. We hope that MAXS-based structures with "dynamics" structure will be utilized for the elucidation of the catalytic and regulatory mechanisms of proteins and new drug discovery strategies in the future. We hope that you will feel the arrival of the era of structures with "movement" beyond "stationary" structures.

We have created movies of the structural ensemble models of the apo form, the binary complex, and the ternary complex described in this article. We hope you will watch these videos and feel the importance of "dynamics".

(Structural ensemble model video: https://japan.rigaku.com/biomaxs/video/Apo-vibes_RigakuLogo.mp4)

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