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High-resolution structure discloses the potential for allosteric regulation of mitogen-activated protein kinase kinase 7

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ABSTRACT

Mitogen-activated protein kinase kinase 7 (MAP2K7) regulates stress and inflammatory responses, and is an attractive drug discovery target for several diseases including arthritis and cardiac hypertrophy. Intracellular proteins such as MAP2K7 are prone to aggregation due to cysteine-driven oxidation in *in vitro* experiments. MAP2K7 instability due to the four free cysteine residues on the molecular surface abrogated the crystal growth and led to a low-resolution structure with large residual errors. To acquire a higher resolution structure for promoting rational drug discovery, we explored stable mutants of MAP2K7 by replacing the surface cysteine residues, Cys147, Cys218, Cys276 and Cys296. Single-site mutations, except for Cys147, maintained the specific activity and increased the protein yield, while all the multi-site mutations massively reduced the activity. The C218S mutation drastically augmented the protein production and crystallographic resolution. Furthermore, the C218S crystals grown under microgravity in a space environment yielded a 1.3 Å resolution structure, providing novel insights for drug discovery: the precisely assigned water molecules in the active site, the double conformations in the flexible region and the C-terminal extension bound to the N-terminal region of the adjacent molecules. The latter insight is likely to promote the production of allosteric MAP2K7 inhibitors.

1. Introduction

Mitogen-activated protein kinase kinase 7 (MAP2K7) is involved in the c-Jun N-terminal kinase (JNK) signaling cascade, which mainly regulates stress and inflammatory responses [1]. MAP2K7 is an attractive drug discovery target for several diseases including arthritis, hepatoma and cardiac hypertrophy [2–4]. The selectivity of MAP2K7 inhibitors towards other kinases, in particular MAP2Ks (MAP2K1–MAP2K6), is a primary barrier in the drug discovery process. High-resolution crystal structures of MAP2K7 could overcome this issue. Previously, we have obtained the wild type structure of MAP2K7 at a low resolution of 3.0 Å [5,6]. Our crystallization experiments yielded heavy precipitation and membrane-formation, suggesting that chemical instability disturbed the crystal growth of MAP2K7. Four free cysteine residues, Cys147, Cys218, Cys276 and Cys296 (Fig. 1), on the surface likely promote this oxidative aggregation. Cys147 resides in the glycine-rich loop (P-loop) involved in ATP accommodation (Fig. 1). Cys218 at the N-terminal of the αD helix and Cys276 in the deep ATP-binding site hardly have direct contribution to the ATP binding (Fig. 1). Cys296 locates in the activation loop (A-loop), serving as a substrate peptide-binding platform (Fig. 1). Previously, we have reported that the replacement of the free cysteines on the molecular surface largely improved the stability of JNK [7]. The single mutation, C218S, manipulated for the validation of the auto-inhibition mechanism of MAP2K7, stabilized the protein, resulting in a 2.1 Å resolution structure [6]. Here, we surveyed the effect of single or multiple cysteine replacements on the stabilization of MAP2K7. Furthermore, microgravity environment was applied to the crystal growth process, to improve the crystal quality of the most stabilized MAP2K7 mutant. The crystallization procedure under microgravity at the International Space Station has been established by Japan Aerospace Exploration Agency (JAXA) and

Abbreviations: MAP2K, mitogen-activated kinase kinase; JNK, c-jun N-terminal kinase; DVD, domain for versatile docking.

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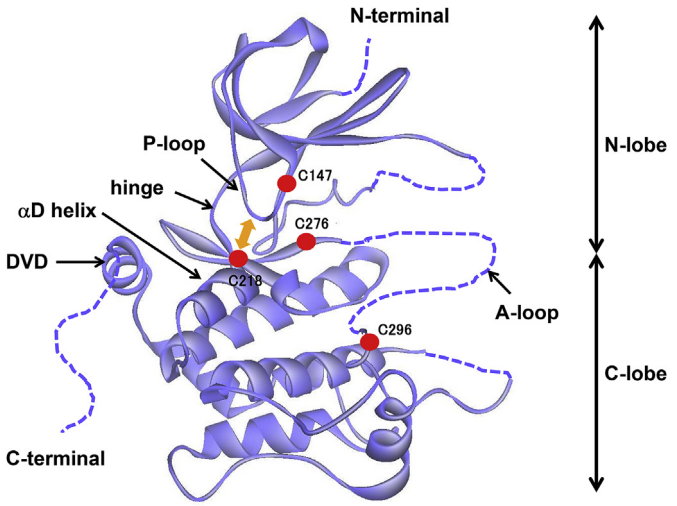

Fig. 1. The overall structure of wild type MAP2K7 depicted the auto-inhibition state. This configuration is established by the glycine-rich loop referred to as P-loop, which intrudes into the ATP site and interacts with Cys218 (orange arrow). Dashed lines present the disordered region in the crystal structure. The positions of the replaced cysteine residues are shown by red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

successfully applied to several proteins such as human triosephosphate isomerase [8]. Finally, we achieved a well-defined crystal structure at 1.3 Å resolution, which unveiled a novel drug discovery approach for producing selective MAP2K7 inhibitors.

2. Material and methods

2.1. Protein preparation

Wild type MAP2K7 was prepared as previously reported [5,6]. Hexa-histidine-tagged MAP2K7 was produced by transforming the *Escherichia coli* strain, Rossetta2 (DE3) pLysS (Merck Millipore, Darmstadt, Germany), with the *MAP2K7* gene inserted into pET22b (Merck Millipore). The protein was purified by TALON cobalt-charged resin (Takara Bio, Otsu, Japan) and SP Sepharose FF cation-exchange column (GE Healthcare, Little Chalfont, UK). The expression vector for the mutant was generated by site-directed mutagenesis, using the wild type *MAP2K7* gene as the template. The mutant proteins were prepared by the same procedure as the wild type protein.

2.2. Cascade kinase assay

Cascade kinase reactions were conducted in a solution containing active mitogen-activated protein kinase kinase kinase 3 (MAP3K3) (Carna Biosciences, Kobe, Japan) as the client kinase of MAP2K7, inactive MAP2K7 prepared by the above procedure (wild type or mutant), unphosphorylated GST-tagged JNK1 (Carna Biosciences) as the substrate, 20 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ and 25 μM ATP at room temperature. Phosphorylated JNK1 was detected by an anti-phospho-pJNK1 monoclonal antibody (Merck Millipore) as the primary antibody on a glutathione-coated ELISA plate (Thermo Fisher Scientific, Waltham, MA, USA). The amount of immunocomplexes was detected using TMB chemical luminescence evoked by horseradish peroxidase conjugated to a goat anti-mouse IgG antibody (Thermo Fisher Scientific) as the secondary antibody. The IC_{50} was determined by PRISM6 (GrafPad Software, San Diego, LA, USA).

2.3. Crystallization and structural analysis

The cysteine mutants of MAP2K7 were crystallized under the same conditions as the wild type, consisting of 22–25% (w/v) PEG3350, 0.2 M sodium citrate tribasic and 0.1 M HEPES buffer, pH 7.5 [5,6]. Under a microgravity environment, crystals were obtained at 277 K, using the counter-diffusion method [9] in the Japanese Experimental Module "Kibo" at the International Space Station (ISS) [10]. The X-ray diffraction data was collected on a Quantum 270 (ADSC) at the Photon Factory BL17A beamline and integrated by HKL2000 [11]. The initial phase was determined by molecular replacement using the 5B2K or 5B2L structure [6] as a starting model. Structure refinement and model modification were iterated using Refmac5 [12] and Coot [13] in the CCP4 program suite [14]. The data collection and refinement statistics are shown in Table 1. The final coordinates of the C218S and C276S mutants were deposited in the Protein Data Bank with IDs: 5Y90 and 5Y8U, respectively.

2.4. Differential scanning calorimetry (DSC)

The thermal unfolding of MAP2K7 was measured by Nano DSC (TA Instruments Inc., New Castle, DE, USA) using platinum tubing cells with a volume of 0.3 ml. The wild type protein or C218S mutant was concentrated to 1.7 mg/ml and 2.7 mg/ml, respectively, in an experimental buffer containing 250 mM NaCl and 20 mM phosphate, pH 7.0. Temperature scans were performed from 0 to 70 °C at a scan rate of 60 °C/h.

3. Results and discussion

3.1. Cysteine replacement effects on stability and enzyme activity

The overall protein yield after purification is a good indicator of the stabilization level of the cysteine replacement mutant compared with the wild type. The cysteine replacement mutation on the molecular surface allowed a higher yield and chemical stabilization of JNK1 [7]. The single cysteine replacements, except for C147S, increased the protein production (Table 2) and likely

Table 1
Data collection and refinement statistics.

	C276S	C218S
Data collection		
Space group	$P6_122$	$P2_12_12_1$
Unit cell (Å)	$a = b = 71.53$	$a = 52.396$
	$c = 262.31$	$b = 70.607$
		$c = 92.155$
Observations	190253	544892
Unique reflections	9446	84643
Resolutions (Å)	61.95–2.92 (2.97–2.92)	56.05–1.30 (1.32–1.30)
Completeness (%)	100 (100)	100 (100)
R_{merge} (%)[a]	9.5 (80.2)	2.8 (87.8)
I/σ	34.7 (4.1)	60.4 (1.9)
Refinement statistics		
Resolution (Å)	61.95–2.92 (2.99–2.92)	56.05–1.30 (1.33–1.30)
Reflections	9376	77503
Total atoms	2137	2925
R-factor (%)	23.0 (27.4)	12.7 (19.1)
R_{free} (%)	29.9 (39.1)	16.7 (25.2)
R.m.s. deviations		
Bond length (Å)	0.012	0.023
Bond angle (°)	1.7	2.1

Values in parentheses are for the highest-resolution shell.
[a] $R_{merge} = \Sigma_h\Sigma_j|I_{hj}-<I_h>|/\Sigma_h\Sigma_j|I_{hj}|$, where h represents a unique reflection and j represents symmetry-equivalent indices. I is the observed intensity and $<I>$ is the mean value of I.

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