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RESEARCH ARTICLE

HIGH PRESSURE PROMOTES BINDING OF THE ALLOSTERIC INHIBITOR Zn^{2+} -CYCLEN IN CRYSTALS OF ACTIVATED H-RAS

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In memory of Professor Roger Fourme who was a pioneer in high pressure macromolecular crystallography (HPMX)

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Abstract: In this work, we experimentally investigate the potency of high pressure to drive a protein toward an excited state where an inhibitor targeted for this state can bind. Ras proteins are small GTPases cycling between active GTP-bound and inactive GDP-bound states. Various states of GTP-bound Ras in active conformation coexist in solution, amongst them, state 2 which binds to effectors, and state 1, weakly populated at ambient conditions, which has a low affinity for effectors. Zn^{2+} -cyclen is an allosteric inhibitor of Ras protein, designed to bind specifically to the state 1. In H-Ras(wt).Mg²⁺.GppNHp crystals soaked with Zn^{2+} -cyclen, no binding could be observed, as expected in the state 2 conformation which is the dominant state at ambient pressure. Interestingly, Zn^{2+} -cyclen binding is observed at 500 MPa pressure, close to the nucleotide, in Ras protein that is driven by pressure to a state 1 conformer. The unknown binding mode of Zn^{2+} -cyclen to H-Ras can thus be fully characterized in atomic details. As a more general conjunction from our study, high pressure x-ray crystallography turns out to be a powerful method to induce transitions allowing drug binding in proteins that are in low-populated conformations at ambient conditions, enabling the design of specific inhibitors.

Introduction

The oncogene Ras protein is a small GTPase involved in many cancers, making this protein an interesting therapeutic target.^[1-4] The catalytic domain of Ras is highly conserved, with a Rossman fold domain composed of 6 β -strands and 5 α -helices. Its structure can be described as bi-lobe with a N-terminal effector lobe containing the active site and the effector binding region and a C-terminal allosteric lobe containing the membrane interacting regions (Figure S1). Three isoforms exist in mammals, H-Ras, K-Ras and N-Ras.^[5-7]

Ras proteins cycle between three states, an inactive state when complexed with GDP, a transient nucleotide-free state, and an active state when complexed to GTP. The activation/deactivation processes are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Two specific segments of Ras termed switch I (amino acids 29-40) and switch II (amino acids 60-76) undergo significant conformational change upon nucleotide exchange. These two flexible segments are also involved in effectors binding, which leads to activation of downstream signal to the nucleus. Most Ras mutants at sequence positions 12, 13, and 61 are strongly

RESEARCH ARTICLE

oncogenic. In the case of H-Ras the medically most significant mutations are G12S, G13R, and Q61R, and in the case of K-Ras they are G12D, G12V, G12C and G13D. Most of these mutants decrease the intrinsic GTPase activity somewhat^[8]; however, the biologically dominant effect is the inhibition of the GTPase activity catalyzed by GAP, leading to constitutive activation of Ras effector pathways^[9,10]. Mutations of Ras are found in nearly 30 % of human tumors.^[2,9,11]

The discovery of potent inhibitors of Ras are still challenging. Several compounds have been described that bind to Ras and inhibit signal transduction.^[12–20] Oncogenic K-Ras mutants have been targeted by T-cell transfer therapy and by PROTACs.^[21,22] However, a really effective therapy targeting Ras has not emerged until now.

Ras proteins adopt many conformational states with a dynamic equilibrium between them. The two major GTP-bound states (T) are the inactive state 1(T) where the switch I adopts an open conformation and the catalytically active state 2(T) where the switch I adopts a close conformation over the nucleotide. The state 2 represents the effector binding state, recognized by effector proteins such as Raf kinases, while the state 1 which is ready for GEF-binding, has a very low affinity for effectors^[10,23–25]. At ambient conditions, state 2 is the dominant state while state 1 is weakly populated.

Pressure is an ideal tool to explore the conformational landscape of biological macromolecules since it modifies the equilibrium between different conformers that coexist in solution. High pressure increases the population of conformers with smaller partial volumes which correspond to excited states. These excited states may be functionally relevant and involved in interaction with partners.^[26–28] The effect of high hydrostatic pressure can be investigated by high pressure NMR^[28–30] and high pressure crystallography.^[31–37]

High pressure NMR on Ras has revealed that pressure modifies the conformational equilibrium in solution between the two main states and induces a reduction in the state 2 population in favor of the state 1 population in active Ras.^[38,39]

High pressure crystallography on Ras has revealed that application of pressure on H-Ras(wt).Mg²⁺.GppNHp Ras crystals in the main state 2 conformation induces above 300 MPa, an *in-crystallo* phase transition allowing the slow switch from one conformational state to another.^[39]

State 2 was considered as “undruggable” for a long time while the state 1 possesses druggable pockets located between switch I and switch II, which do not exist in the state 2 conformer, being filled by amino acids of switch I.^[18,40]

Inhibitors that target specific state of Ras have been identified, one of the first being the Zn²⁺-cyclen. Cyclen (1,4,7,10-tetraazacyclododecane) forms strong complexes with divalent ions such as Zn²⁺ or Cu²⁺ and trivalent ions such as Gd³⁺. Gd³⁺-Dota (a cyclen derivative) is approved as paramagnetic contrast agent in MRI since 1998. Zn²⁺-cyclen is interacting with nucleic acids and has been described as artificial nuclease^[41]. In 2005, it was shown that Zn²⁺-cyclen specifically binds to activated Ras in the GEF-binding state 1, the low affinity state of Ras for effectors, and thus inhibits Ras signal transduction^[42]. The interaction of Zn²⁺-cyclen follows cooperative binding. Stabilizing an inactive

conformation at expense of state 2 is a general mechanism (“intrinsic allosteric inhibition”) for impairing Ras-effector association and thus the signal transduction.^[43–45]

In the present study, we have investigated the high-pressure crystallographic structures of H-Ras(wt).Mg²⁺.GppNHp catalytic domain (residues 1-166) soaked with Zn²⁺-cyclen at 0.1 MPa, 200 MPa and 500 MPa. No binding of Zn²⁺-cyclen could be observed at 0.1 MPa and 200 MPa, as expected in the state 2 conformation which is the dominant state at ambient or low pressure. Very interestingly, at 500 MPa, we were able to localize the Zn²⁺-cyclen binding site close to the nucleotide in Ras protein entirely in state 1, i.e. the conformational state with reduced affinity for effector partners.

We thus demonstrate that high pressure macromolecular crystallography allows to fully characterize the drug binding site in a conformational state of Ras rare at ambient pressure.

Results

The structures of H-Ras(wt).Mg²⁺.GppNHp and H-Ras(wt).Mg²⁺.GppNHp in complex with Zn²⁺-cyclen will be referred in the following as ligand-free Ras and Ras-cyclen respectively. The crystallographic structures of Ras-cyclen at ambient pressure, 200 MPa and 500 MPa were diffracting at 1.80 Å, 2.05 Å and 2.5 Å respectively (Table S1).

Similar to the results on ligand-free Ras crystals,^[39] we observed with Ras-cyclen crystals an *in-crystallo* phase transition between 200 MPa and 500 MPa.

The crystallographic structure of Ras-cyclen at 200 MPa is quite similar to the one at 0.1 MPa, with a root mean square deviation (r.m.s.d) on C α of 0.17 Å. However, the main chain thermal B-factors strongly increase (by 7.5 Å² in average), as it was observed in ligand-free Ras.^[39] No Zn²⁺-cyclen molecules could be located in the electron density map at ambient pressure and at 200 MPa.

The structure of Ras-cyclen at 500 MPa becomes different from the structure at 200 MPa before the phase transition, with a r.m.s.d on C α of 0.84 Å. The helix α 2 (which corresponds to the switch II) is shifted toward helix α 3. In particular, a direct hydrogen bond between the side chains of Ser 65 and Gln 99 is now established, and the distance between Arg 68 and Tyr 71 side chains is decreased. Moreover, the switch I between Val 29 and Ser 39 adopts an open conformation, with a marked deviation by more than 5 Å away from the nucleotide (Figure 1).

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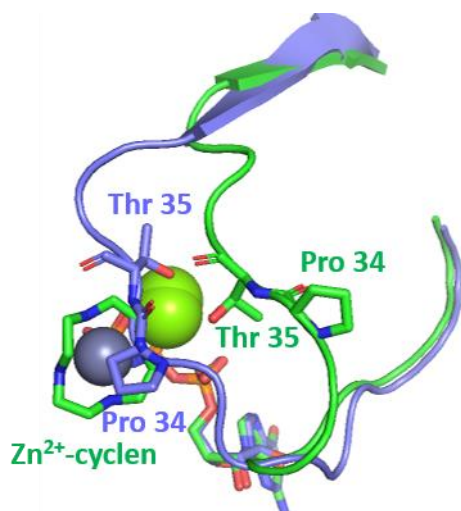


Figure 1. The switch I of Ras-cyclen structure at ambient pressure (in slate) and at 500 MPa (in green) highlighting its large shift. The two residues Pro 34 and Thr 35, the GppNHp and cyclen molecules are shown in stick representation colored by atom types, the Mg^{2+} and Zn^{2+} ions as green and grey spheres respectively.

Very interestingly, a Zn^{2+} -cyclen molecule is clearly located in the electron density map, in interaction with the nucleotide, with a distance from the Zn^{2+} -ion to the γ -phosphorus atom of 3.20 Å (Figure 2). The Zn^{2+} -cyclen molecule interacts with the Thr 35 and Gln 61 side chains, two of the key residues in Ras cycle, even if the precise conformation of the cyclen ligand cannot be unambiguously determined.

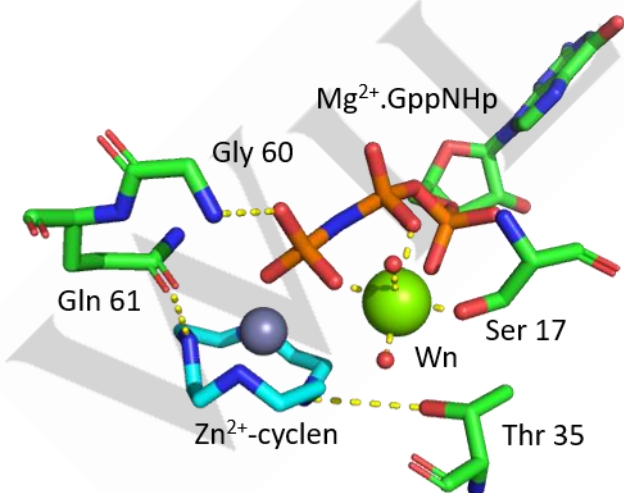


Figure 2. The Zn^{2+} -cyclen molecule is bound close to the nucleotide in the Ras-cyclen structure at 500 MPa. The residues Ser 17, Thr 35, Gly 60, Gln 61, and the GppNHp and cyclen molecules are shown with stick colored by atom types with C in green (cyan for cyclen molecule), O in red, N in blue and P in orange. The Zn^{2+} and Mg^{2+} ions and water molecules are shown with spheres whose radius have been reduced for clarity.

In the crystal packing, the Zn^{2+} -cyclen molecule lies parallel to its symmetric mate of the neighboring Ras protein, with an occupation of 0.5 each. The two symmetric molecules cannot be present simultaneously, being too close to each other (Figure 3).

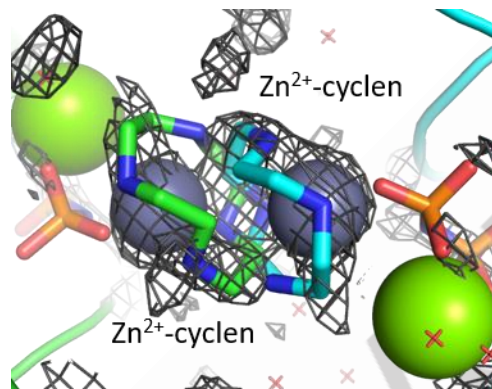


Figure 3. Omit electron density map around the Zn^{2+} -cyclen molecule contoured at 2σ , with the two symmetry mates colored with C atoms colored in green and cyan, respectively. The GppNHp and cyclen molecules are shown in stick representation colored by atom types, and the Zn^{2+} and Mg^{2+} ions are depicted with grey and green spheres, respectively.

Discussion

Applying a pressure above the pressure that induces an *in-crystallo* phase transition allows binding of Zn^{2+} -cyclen molecule. Its location close to the nucleotide in Ras-cyclen crystallographic structure at 500 MPa is in agreement with paramagnetic relaxation enhancement and chemical shift perturbation NMR experiments which indicated that the divalent ion of the cyclen molecule interacts directly with the γ -phosphate of the nucleotide in Ras state 1, with a distance of around 3 Å.^[43]

A second Zn^{2+} -cyclen binding site was previously identified close to the C-terminal His 166, far from the nucleotide, by crystallography on Ras crystals soaked with Zn^{2+} -cyclen belonging to the H32 space group and by chemical shift perturbation NMR experiments.^[43] In the present study, Ras crystals belong to the P3₂21 space group and the crystal packing prevents binding near the C-terminal histidine.

The crystallographic structures of Ras-cyclen at ambient pressure and at 200 MPa are very similar to previous crystallographic structures of H-Ras(wt) catalytic domain in complex with a GTP analog Mg^{2+} .GppNHp, with the switch I in close conformation over to the nucleotide^[46–48] These two Ras-cyclen structures correspond to the dominant state 2 conformation where the Thr 35 hydroxyl group coordinates the Mg^{2+} -ion. The absence of Zn^{2+} -cyclen confirms that this molecule cannot bind near the nucleotide when Ras is in state 2 conformation.

In the crystallographic structures of Ras-cyclen at 500 MPa, the switch I adopts an open conformation away from the nucleotide. In this conformer, the hydroxyl group of Thr 35 is thus no longer coordinated to the Mg^{2+} ion and is replaced by a water molecule termed here Wn (Figure 2). The position of Zn^{2+} -cyclen

RESEARCH ARTICLE

corresponds indeed to Pro 34 and Thr 35 positions as observed in all Ras structures in state 2 conformation (Figure 1). The structure of Ras-cyclen at 500 MPa is then quite similar to previously described Ras structures representative of the state 1, like Ras mutants T35S or G60A, a glue-coated wild-type Ras prepared in humid air (HAG), or a WT Ras whose crystals were grown by seeding approach with seeds generated from T35S crystals,^[49–51] where the switch I adopts an open conformation and the interaction between Thr 35 and Mg²⁺-ion is no longer present. These similarities highlight that the Ras-cyclen structure at 500 MPa clearly adopts a state 1 conformation. This study then confirms that Ras has to be in state 1 conformation to be targeted by an allosteric inhibitor such as Zn²⁺-cyclen.

In the different structures of state 1 WT or mutants already described^[49–51], there is a large positional change of Gln 61 side chain away from the nucleotide, toward Arg 68 and Gln 99 side chains, which does not occur in Ras-cyclen structure at 500 MPa. In this structure, the switch I has shifted to an open conformation away from the nucleotide while there is no conformational modification in the switch II. This structure may thus represent an intermediary state between a state 2 structure and a full state 1 structure. In analogy to the solution NMR studies, the Ras-structure with cyclen bound should represent an 1(T)* structure which originates from an induced fit of the state 1(T) structure after cyclen binding^[45].

The Zn²⁺-cyclen is bound close to the nucleotide in a surface pocket which does not exist in structures of Ras in state 2, confirming that only state 1 possesses druggable pockets (Figure 4).^[18,40]

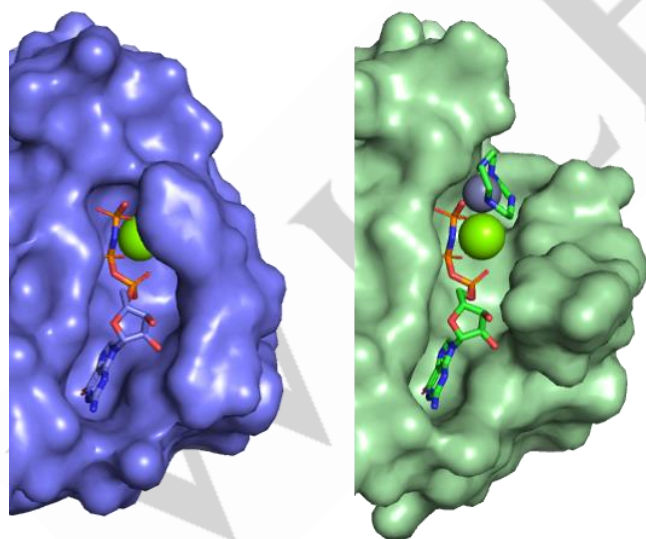


Figure 4. Ras-cyclen structures shown with their molecular surfaces at 0.1 MPa (left) and at 500 MPa (right) with the GppNHp molecule and the cyclen molecule shown in stick and the Mg²⁺ and Zn²⁺ ions shown as spheres.

The crystallographic structures of Ras-cyclen at 0.1 MPa and at 200 MPa do not significantly differ from the structures, at identical pressure, of ligand-free Ras, which are in state 2 conformation.

Indeed, the r.m.s.d on C α are of 0.12 Å and 0.18 Å, respectively. This suggests that the soaking with Zn²⁺-cyclen does not significantly alter the Ras structure, nor the conformation equilibrium.

At 500 MPa, with the exception of switch I (Val 29 – Tyr 40), the behavior upon pressure of Ras-cyclen is similar to ligand-free Ras, confirming that the equilibria between allosteric states do not depend on the presence of Zn²⁺-cyclen (Figure S2A). The structural changes located in the β 2- β 3 loop (Gln 43 – Cys 51) and the switch II (Gly 60 – Tyr 71) which are shifted by more than 1 Å, are similar to those found in the structural transition between 0.1 MPa and 500 MPa in ligand-free Ras. In particular, 27 residues share identical structural modifications when comparing the structures at 500 MPa (with or without cyclen) with the structures at 200 MPa or 0.1 MPa (with or without cyclen).

However, only a slight decrease of main chain B-factors could be observed at 500 MPa compared to 0.1 MPa, contrary to what was observed in ligand-free Ras transition (Figure S2B).^[39] This may directly be related to Zn²⁺-cyclen binding and switch I movement and may also be indicative of a cooperative effect of Zn²⁺-cyclen in facilitating the transition toward state 1 after its initiation by high-pressure.

We previously shown that increasing the pressure on Ras crystals between 200 MPa and 500 MPa induces a transition of the segment between Asp 69 – Val 109 from a state 2 to a state 1^[39] which leads to a slightly increased separation between the switch I and the switch II, more precisely between the three first β -strands and helix α 2. The conformational change of this segment would enable the allosteric inhibitors to bind.

Interestingly, in several previously determined structures of GTP-bound-Ras mutants with inhibitors bound in a pocket termed SI/II, located between the two switches,^[16,17,40,52–54] an enhanced separation between the three first β -strands and helix α 2 is also observed, highlighting that in these structures, the segment Asp 69 – Val 109 is also in the state 1. This SI/II-pocket was shown to have a high druggability score.^[55]

These similarities in the segment Asn 69 – Val 109 in different structures of Ras with inhibitors bound either in the SI/II-pocket as for indole-like inhibitors or in the nucleotide binding site as in the case of Zn²⁺-cyclen unambiguously show that at least the transition of this segment to state 1 is required for the binding of allosteric inhibitors targeting this conformational state. This transition is mandatory, whatever the inhibitor interaction site involves the nucleotide or the SI/II pocket.

It was also noticed that when an inhibitor is bound in this SI/II pocket, Tyr 71 is buried within the protein core.^[17,55] We have previously highlighted that this tyrosine is a sensor of the conformational state of Ras, toggling by almost 180° between a position toward the bulk and a position toward the core.^[39] In the structure of Ras-cyclen at 500 MPa, Tyr 71 is indeed oriented toward the core, while it was oriented toward the bulk in the structure at ambient pressure.

The Ras-cyclen structure at 500 MPa is to our knowledge the first representative of a H-Ras wild-type state 1 crystal structure with an allosteric inhibitor bound, since the previous reported structures with an inhibitor bound in S/II pocket were H-Ras

RESEARCH ARTICLE

mutants, such as T35S mutant which is intrinsically in state 1 or G12D mutant.^[16,17,52,55]

The precise characterization of the Zn²⁺-cyclen binding pocket close to the Mg²⁺-ion in the crystallographic structure of the complex at high pressure as well as the central role of the segment Asn 69 – Val 109 in the 2 - 1 state transition will allow rational design of new compounds with higher affinity than Zn²⁺-cyclen.

This study demonstrates the potency of high pressure to drive a protein toward an excited state where a functional partner can bind. This constitute a proof of concept that the use of high pressure crystallography opens up new possibilities to study at atomic resolution drug binding to rare conformational states of proteins. Development of new drugs targeting specific pocket of different Ras isoforms, specially K-Ras and mutants which are highly oncogenic,^[56,57] would take advantage of high pressure crystallography to determine high resolution structures of high energy conformers, to visualize with great details ligand binding site in these excited states and enable more efficient ligand screening.

Experimental section

Protein expression and purification

Truncated variant (amino acids 1-166) of H-Ras(wt) was expressed in LB medium in the *E. coli* strains CK600K using the pTac vector under the control of the *lac* operon. Protein was purified by anion exchange chromatography, using a Q-Sepharose column, followed by size exclusion chromatography, using a Superdex 26/600 200 PG column (GE Healthcare, München, Germany). For more details see Girard *et al.*^[39]

Nucleotide exchange

Nucleotide exchange from GDP to GppNHp was subsequently performed at 278 K in a Mg²⁺-free buffer with addition of 200 mM (NH₄)₂SO₄ on a column with immobilized alkaline phosphatase (MoBiTec, Göttingen, Germany) in the presence of a 4-fold excess of GppNHp. After the reaction, free nucleotides, salt and other reaction by-products were removed by size exclusion chromatography using a PD10 column (Amersham, Freiburg, Germany). The final product, 1 to 1.5 mM H-Ras.Mg²⁺-GppNHp dissolved in 40 mM Tris/ HCl pH 7.5, 10 mM MgCl₂, 2 mM DTE (buffer A), was frozen in aliquots and stored at 193 K. For more details, see Girard *et al.*^[39]

Crystallization

Ras crystals were produced in the batch mode by mixing 30 uL of buffer A with 30 uL of 80 mM Tris HCl pH 7.5, 20 mM MgCl₂, 4 mM DTE and 52-60% PEG-400 (buffer B). The two solutions were mixed by gentle pipetting and the crystallization chambers immediately sealed with transparent adhesive tape and placed in a vibration-free zone in the dark. Full-sized crystals in trigonal space group P3₂21 were grown after 2-3 days. The already grown crystals were soaked in 8-12 mM of Zn²⁺-cyclen dissolved in the

crystallization buffer B, for at least 10 hours prior to the experiments.

High pressure macromolecular crystallography (HPMX)

Ras(wt)- Zn²⁺-cyclen crystals were loaded into a diamond anvil cell (DAC) as previously described,^[39,58,59] using the crystallization buffer supplemented with Zn²⁺-cyclen as compression media. We used two large-aperture DACs designed by Roger Fourme and colleagues and manufactured by BETSA® (Nangis, France). The pressure within the DAC compression chamber was monitored through the pressure-dependent fluorescence of a ruby chip used as an internal probe. Exposure time was 10 s per frame with an oscillation angle of 1°. Diffraction data were recorded at room temperature on the CRISTAL beamline at the synchrotron SOLEIL (Saint-Aubin, France) at wavelength $\lambda = 0.51035 \text{ \AA}$ ($E = 24.29 \text{ keV}$), with a Rayonix SX-165 detector. Data collection at short wavelengths reduces the absorption of the diamond windows and increases the accessible reciprocal space, allowing higher resolutions to be reached.^[60] During data collection, the crystal was translated in the beam every 10° of rotation to limit the crystal degradation by irradiating fresh portions of the crystal.

Data processing

3 data sets have been collected in the condition described in Girard *et al.*,^[39] a reference data set at ambient pressure, a data set at 200 MPa, then a data set at 500 MPa on a new crystal that was first pre-incubated overnight at 200 MPa. All data sets were indexed and integrated using XDS.^[61] The integrated intensities were scaled and merged using SCALA and TRUNCATE from the CCP4 package.^[62] The structures were solved by molecular replacement (MR) with PHASER^[63] from the PHENIX package.^[64] In order to minimize any bias in the areas involved in the transition between states, MR's were performed using a model from the PDB file 5p21 where the switch I (Asp 30 - Arg 41) and switch II (Gly 60 - Glu 76) were removed in addition to all heteroatoms and alternate positions. The three data sets were refined with PHENIX^[65] starting from the MR model, however the Ras-cyclen structure at 500 MPa has been refined with group B-factors. The space group has been checked with ZANUDA from the CCP4 package. In all cases, the resulting models were analyzed and rebuilt with the graphical tool COOT.^[66]

The C α root mean square deviations (r.m.s.d.) and the average backbone thermal B-factors were computed using LSQKAB and BAVERAGE, respectively, from the CCP4 package. A summary of data collection and refinements statistics is reported in Table S1. The figures have been made using PyMOL (Version 2.4.0 Schrödinger, LLC).

Data availability

The atomic coordinates and structure factors have been deposited in the Protein Data Bank with ID numbers 8OSN, 8OSM, 8OSO for Ras-cyclen at 0.1 MPa, 200 MPa and 500 MPa respectively.

Supporting Information

RESEARCH ARTICLE

Supplementary figures and table are shown in the Supporting Information.

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Keywords: • allosterism • drug design • high pressure crystallography HPMX • Ras protein • in crystallo transition

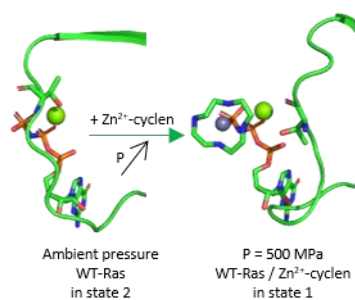
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RESEARCH ARTICLE

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Entry for the Table of Contents



High pressure induces in the crystal of Ras(wt).Mg²⁺.GppNHp, which is in the dominant state 2 conformation, a transition to a transient state where the Zn²⁺-cyclen molecule, which is an allosteric inhibitor targeting the state 1, can bind.