Structural and functional characterization of autophosphorylation in bacterial Histidine Kinases

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Abstract

Autophosphorylation of histidine kinases (HK) is the first step for signal transduction in bacterial two-component signalling systems. As HKs dimerize, the His residue is phosphorylated in *cis* or *trans* depending on whether the ATP molecule used in the reaction is bound to the same or the neighbouring subunit, respectively. The *cis* or *trans* autophosphorylation results from an alternative directionality in the connection between helices $\alpha 1$ and $\alpha 2$ in the HK DHp domain, in such a way that $\alpha 2$ could be oriented almost 90° counter-clockwise or clockwise with respect to $\alpha 1$. Sequence and length variability of this connection appears to lie behind the different directionality and is implicated in partner recognition with the response regulator (RR), highlighting its importance in signal transduction. Despite this mechanistic difference, HK autophosphorylation appears to be universal, involving conserved residues neighbouring the phosphoacceptor His residue. Herein, we describe a simple protocol to determine both autophosphorylation directionality of HKs and the roles of the catalytic residues in these protein kinases.

Keywords: Histidine kinases, two-component systems, *cis-trans*-autophosphorylation, signal transduction, heterodimer production and purification, X-ray crystallography.

1. Introduction

Bacterial histidine kinases (HKs) are signal transduction enzymes involved in the sensing of different stimuli and transmitting changes in those stimuli to the inner cell [1]. Sensing and transmitting signals by HKs imply ATP uptake, autophosphorylation and phosphoryl transfer. In recent years, autophosphorylation of HKs has drawn attention as the structural and functional features governing this enzymatic activity have been better characterised. HKs are dimeric proteins and comprise a catalytic core formed by two domains joined by a flexible linker. One domain, called DHp (Dimerization and Histidine phosphotransfer), is formed by two antiparallel α -helices ($\alpha 1$ and $\alpha 2$) connected by a loop, while the other domain, CA (Catalytic and ATP-binding), is globular with an α/β sandwich fold that binds ATP [2, 3] (Figure 1). The DHp domain holds the phosphoacceptor His at helix $\alpha 1$ and mediates dimerization into a four helix bundle merging two subunits into a dimer (Figure 1). In that way, the CA domain that binds ATP can approach the phosphoacceptor His in either subunit of the dimer, enabling autophosphorylation [4]. If the CA domain bound to ATP approaches the phosphoacceptor His within the same subunit, the HK adopts the so called “*cis*” autophosphorylation conformer, while if it approaches the His of the other subunit the HK adopts the “*trans*” autophosphorylation conformer [5-7] (Figure 1). Structural and functional evidence suggests that the connection between helices $\alpha 1$ and $\alpha 2$ in the DHp domain could play a key role regulating the directionality of autophosphorylation [7, 8, 9]. Therefore, it has been hypothesized that the length and residue composition of this link (Figure 2) would select between *cis* or *trans* autophosphorylation [10]. Crystal structures of DHp domains from HKs whose autophosphorylation has been confirmed

biochemically to occur in *cis* (*i.e.* HK853 from *Thermotoga maritima* [7]) or in *trans* (*i.e.* EnvZ and CpxA from *E. coli* [11, 12]) show that the helix $\alpha 2$ in the DHp domain is connected almost 90° counter-clockwise or clockwise with respect to $\alpha 1$, respectively (Figure 3). Interestingly, the alternative directionality adopted by HKs is independent of the catalytic mechanism for the autophosphorylation reaction, which has been demonstrated to be conserved [10]. Thus, the change in directionality at the connection between helices $\alpha 1$ and $\alpha 2$ could play a different role in two-component systems (TCS) signalling. Since the DHp $\alpha 1$ - $\alpha 2$ connection is one of the regions recognized by the RR in the interaction with the HK, alternative directionalities could be critical in preserving the exquisite fidelity showed by the HK-RR pairs in TCS, avoiding cross-talk between phosphate donors and acceptors, which could compromise bacterial survival. Here, we will outline methodology that can be used to unveil if an HK works by a *cis* or *trans* autophosphorylation reaction, which should be directly related with the directionality of the connection between helices $\alpha 1$ and $\alpha 2$ at the DHp domain. Moreover, we will dissect the catalytic mechanism for the autophosphorylation reaction through the analysis of the most relevant catalytic residues. These methodologies will be broadly applicable for the characterisation of structural and functional features of other HKs that govern their autophosphorylation.

2. Materials

2.1 Cloning

The proteins used for all the experiments should correspond to the catalytic domains of HKs, comprising only the DHp and CA domains. To locate the catalytic domains of the HK under study, computational tools such as SMART

[13] and Pfam [14] can be used. For example, the catalytic domain of HK853 of *Thermotoga maritima* is comprised from residues 232 to 489, while residues 224-450 constitute the HK of EnvZ from *E. coli*.

1. Chemically synthesized DNA primers to amplify the gene of a HK are prepared at 10 μ M dissolved in water or in Tris-EDTA buffer (TE buffer: 10 mM Tris-HCl pH 8.0 and 1 mM EDTA) (see Note 1 and 2).

2. Bacterial expression plasmids: 5-50 ng dissolved in water or in Tris-EDTA buffer (see Note 3).

3. Agarose gel solution: 1% agarose dissolved in 1x TAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA) and heat in the microwave till completely dissolved. For a 100ml gel add 2 μ L of GreenSafe Premium (NZYtech, Portugal) before loading solution in casting tray with comb.

4. PCR or gel cleaning kit (eg. NucleoSpin Gel and PCR Clean-up, Macherey-Nagel)

5. DpnI restriction enzyme (Clontech).

6. T4-DNA polymerase (Thermo Fisher Scientific) (see Note 4)

7. Competent cells of *E. coli* strain DH5 α ; use 50 μ L of electro-competent cells prepared in 10% glycerol (see Note 5).

8. Luria-Bertani (LB) agar plates: Weigh 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 15 g bacteriological agar and make up to 1 L in water. Autoclave and when the medium is under 50°C add the corresponding antibiotic, typically ampicillin at 100 μ g/ml or kanamycin at 33 μ g/ml; mix well and dispense in agar plates.

9. Primers flanking the plasmid-multicloning site.

2.2 Site-directed mutagenesis

It was performed following the Quickchange method developed by Stratagene (La Jolla, CA).

1. High fidelity DNA polymerase such as KOD Hot Start DNA polymerase (Merck Millipore).

2.3 Protein expression

1. For cell growth, prepare Luria-Bertani (LB) broth: weigh 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl make up to 1 L in water, autoclave and when the medium is under 50°C supplement with corresponding antibiotics (Ampiciline 100µg ml⁻¹, Chloramphenicol 33µg ml⁻¹ and/or Kanamycin 33µg ml⁻¹, depending on the cell strain and the plasmid used in each case).

2. Spectrophotometer to measure OD at 600nm

3. Isopropil-β-D-1-thiogalactopiranósido (IPTG) at 1M stock solution or the corresponding inductor to start the protein expression.

2.4 Protein purification

1. Use ultrasonic processor or emulsifier.

2. Lysis buffer (buffer A): 50mM Tris-HCl pH 8.0 and 500mM NaCl. Use a pH according to the protein pI. Add 1mM of phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity and 1mM of -β-mercaptoethanol to avoid oxidation (see Note 6).

3. Affinity chromatography columns, depending on the tag fused to the protein (e.g. 1ml or 5ml HisTrap columns (GE Healthcare) for Histag-proteins).

4. Elution buffer (buffer B): buffer A supplemented with a specific competitor ligand to dissociate the protein bound to the affinity column (e.g. imidazole at 300-500 mM for HisTrap columns).

5. Gel filtration buffer: 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl

6. Gel filtration column: HiLoad 16/60 Superdex 200 (Ge Healthcare)
7. Perform SDS-PAGE using 15% gel with an acrylamide:bisacrylamide ratio (20:1) for a HK with a MW ~30KDa.
8. Loading buffer: mix 3,55ml of deionized water, 1,25 ml of 0,5M Tris pH6,8, 2,5ml of glycerol, 2ml of 10% SDS and 0,2ml of 0,5% Bromophenol blue; homogenize, take 950µl and add 50µl of β-mercaptoethanol. The ratio of sample:loading buffer is 2:1.
9. Centrifugal filters such as Amicon Ultra (Millipore).
10. ÄKTA Purification System (GE Healthcare).
11. SnakeSkin™ Dialysis Tubing, 3.5K MWCO (ThermoScientific).

2.5 HK phosphorylation

1. Buffer A supplemented with 100mM KCl₂ and 10mM MgCl₂.
2. Mixture of cold (0.1-0.3 mM) ATP and hot (0.1 mCi ml⁻¹) ATP-[γ³²] ATP (3000µCi mmol⁻¹) diluted in deionized water. It is recommended to prepare a 10X cocktail to add same amounts to all samples.
3. Stop loading buffer: SDS-PAGE loading buffer containing 2x SDS detergent and 50mM de EDTA.
4. Gel blotting and drying: use 3MM blotting paper to transfer the gel followed by incubation into a gel drying system under vacuum (Bio-Rad Model 583 Gel Dryers and Hydrotech pump).
5. Phosphorylated proteins are visualized by autorradioagrophy using the specific equipment, for example Fluoro Image Analyzer FLA-5000 (Fuji) and analysed with the associated software (MultiGauge software, Fuji).

2.6 Crystallization and crystal freezing

1. Commercial crystallization screens (at least three). We recommend start with JBScreen Classic HTS I+II (from Jena Bioscience) and MIDAS (from Molecular Dimensions).
2. MRC 2-well or 3-well crystallization plates (Molecular Dimensions) that allow to try crystallization of the protein alone and in the presence of ligands such as ATP , ADP or the non-hydrolyzable ATP analogs AMPPNP or AMPPCP.
3. Liquid nitrogen is used for crystal freezing.
4. Cryo loops for crystal mounting (MiTeGen or Hampton Research)).
5. X-ray data processing programs (for example, IMosflm from CCP4 [15]).
6. Software suites for automated macromolecular structure determination by X-ray crystallography (for example, CCP4 [15] and PHENIX [16] or equivalent).
7. Macromolecular visualization software (Coot [17] and PYMOL [18] or equivalent).

3. Methods

3.1 Determination of the autophosphorylation directionality in HKs

Due to the dimeric nature of HKs, the autophosphorylation reaction could take place within the same (intra-)subunit or between (inter-)subunits driving a *cis* or *trans* phosphorylation in the homodimer, respectively (Figure 1). To find out which directionality is driving a specific HK, it is necessary to discern which subunit is phosphorylated within the homodimer and which is the one that phosphorylates. For this propose, heterodimers containing two subunits with different length, a Short (S) and a Long (L) version of the catalytic domains should be produced. Furthermore, to unambiguously distinguish the phosphorylated subunit in the

dimer, two additional types of mutant heterodimers are required. One type contains a mutation at the CA domain just in the L subunit, impairing ATP-binding ($L^{\emptyset}$; *kinase mutant*), and producing the $L^{\emptyset}S$ heterodimer. The other type contains subunit $L^{\emptyset}$ and a mutation at the phosphoacceptor His in the S subunit ($S^{\emptyset}$; *phosphoacceptor mutant*), impairing its capacity to be phosphorylated, and producing $L^{\emptyset}S^{\emptyset}$ heterodimer (Figure 4A).

3.1.1 Cloning of HKs constructs to generate L and S subunits.

To generate subunits L and S with different length, genes for the specific HKs must be cloned into two plasmids, one containing a tag (e.i. His-tag) and the other without tag (or with tag of different size). Plasmids containing tag, or the longest tag will produce a longer HK protein, defined by L, while the plasmids devoid of tag or with the shortest tag will produce shorter protein defined by S.

1. Amplify the genetic region of a specific HK construct by carrying on a Polymerase Chain Reaction (PCR) according to the protocol recommended for the High fidelity DNA polymerase used (e.g. for 25 μ l mix 5-50 ng of DNA template with the polymerase Buffer 1X, specific designed primers and dNTPs at 0.2 μ M final concentration, 2 mM $MgSO_4$ and 1 U of High fidelity DNA polymerase. Then incubate 10 min 95 $^{\circ}C$ for denaturalization followed by 35 cycles of 30 sec at 95 $^{\circ}C$, 30 sec at primer annealing temperature and extension (30 sec/Kb) at 72 $^{\circ}C$. A final extension of 5-10 min at 72 $^{\circ}C$ could be added. See Note 1.

2. Plasmid linearization will vary depending on the cloning techniques used (e.g. LIC cloning, In-fusion or enzyme restriction), thus, use the appropriate technique.

3. Load the PCR products and linearized plasmids into a 1-2% agarose gel to visualize the correct size for the amplified regions and linearized plasmids. Cut

the corresponding gel bands with a scalpel and purify the DNA with a gel cleaning kit following the manufacture's instructions. Linearized plasmids could also be directly cleaned with a PCR clean-up kit without the electrophoretic step.

4. For LIC cloning, treat with T4 polymerase the amplified genes and linearized plasmid. For a 10 μ l reaction: 5 μ L sample, 2.5mM of corresponding dNTP, 0.1 mg/ml BSA, 1x T4 polymerase buffer, 5mM DTT (1,4-Dithiothreitol) and 0.5 μ L of T4-DNA polymerase. Incubate reaction in PCR machine or thermoblock: 25 min 22°C followed by 20 min 75°C for enzyme inactivation and store at 4°C.

5. Then, mix them in a 2:1 ratio (gene:plasmid), incubate at room temperature (RT) for 5min and put them on ice. For In-fusion cloning, mix amplified genes and plasmids in a 2:1 ratio with 5X In-Fusion HD Enzyme Premix, incubate 50°C for 15 min and put on ice.

6. Immediately after step 4 transform 3 μ l of cloning mixture by electroporation in 50 μ l of DH5 α cells. Then, add 1 ml of cold LB to the cells followed by incubation at 37 °C with shaking during 1 h.

7. Centrifuge the tube at 6000 x *g* for 2 min, discard the media without touching the cells. Resuspend slowly the cells in the same media; spread all on a plate with the corresponding antibiotics and grow overnight (o/n).

8. Check if the cloning was successful by picking the colonies grown in the plate and performing a PCR assay (DNA template comes from the colony) using primers flanking the plasmid-multicloning site. Confirm the correct cloning by sequencing PCR products coming from those colonies which show bands of the appropriated molecular size.

9. For further mutagenesis or protein expression analysis, purify plasmid DNA of the positive colonies.

3.1.2 Mutant design

The chosen point mutations are a key factor in this methodology in order to produce subunits that cannot be phosphorylated and subunits that cannot phosphorylate. To obtain a subunit that cannot be phosphorylated, point mutations at the phosphoacceptor His should be introduced in the plasmids devoid of tag or with the shortest tag which can be defined as constructs S⁰ or named as *phosphoacceptor mutant*. Meanwhile to produce a construct that cannot phosphorylate, mutations that impair ATP binding at the CA domain (see Note 7) could be introduced in the plasmids containing the tag or the longest tag, which can be defined as constructs L⁰ or named as *kinase mutant* (see Note 8).

1. Mutagenesis is performed by the Quickchange method developed by Stratagene (La Jolla, CA). A PCR is carried out with the wild type HKs plasmids and the appropriate primers as described in the section 3.1.1.1 but using longer extension times in order to introduce the mutation while amplifying the whole plasmid.

2. To remove methylated parental plasmid, digest de PCR product with 1 µl of Dnpi at 37 °C for 2 hrs or the time indicated by supplier.

3. Continue with transformation as described in 3.1.1.5.

4. Check if the colonies contain the plasmid with the specific mutation by extracting the DNA and sequencing.

3.1.3 Homo and heterodimer production

To produce homodimer and heterodimer species of a HK, similar steps related with expression and purification are followed. However, in the case of heterodimer production other methodologies, besides the co-expression method,

can be used taking advantage of the spontaneous subunit exchange peculiarity of some HKs (see Note 9) [11].

1. For homodimer production, transform by electroporation 20 ng of the corresponding plasmid in 50 μ L of expression cells. For heterodimer production, co-transform 20 ng of each plasmid in 50 μ L of expression cells. To determine the autophosphorylation directionality using heterodimers, three different species of heterodimers LS, L^ØS and L^ØS^Ø should be generated. For that purpose, three different co-transformations should be performed for each HK using two different plasmids containing the appropriate constructs; i) (L) and (S) containing wild type constructs, ii) (L^Ø) and (S) containing mutation at the long construct, iii) (L^Ø) and (S^Ø) containing mutations at both long and short constructs (Figure 4A). Notice that plasmids for the L and S versions of the protein must confer resistance to different antibiotics (see Note 10).

2. Add 1 ml of cold LB to the cells followed by incubation at 37 °C with shaking during 1 h.

3. Spread 100-200 μ l on a plate with the corresponding antibiotics (plasmid and cells) and grow overnight (o/n).

4. To generate a bacterial glycerol stock for future protein expressions, inoculate one colony in 5 ml of LB with the corresponding antibiotics and grow o/n. Next day, mix 800 μ l of saturated culture with 400 μ l of sterile 50 % glycerol and save it at -80 °C.

3.1.4 Protein expression

1. To recover the bacteria from the glycerol stock, scrape some of the frozen bacteria and streak it into a LB plate with the corresponding antibiotics and incubate at 37 °C o/n.

2. Inoculate one colony in 20 ml LB with appropriate antibiotics and grow o/n in a shaker at 37 °C to obtain a saturated culture.
3. Inoculate 20 ml of o/n saturated culture into 1 L LB (for a 1/50 dilution) supplemented with the appropriate antibiotics.
4. When the culture has reached exponential phase ($OD_{600} \sim 0.6$), protein expression is induced by addition of IPTG (1 ml of 1 M stock solution) or the corresponding inductor.
5. After 3 h of induction at 37 °C, cells are collected by centrifugation at 5000 x *g* for 15 min in a refrigerated centrifuge. Then, discard the supernatant and flash-freeze the pellets in LN₂ and store at -80 °C.

3.1.5 Homodimer purification

Protein purification steps are carried at 4 °C.

1. When ready to proceed with the purification, resuspend the cell pellets in cold lysis buffer (around 40-50 ml for the pellet coming from a 1 L culture). Make sure that the suspension is completely homogenized with no clumps (see Note 11).
2. Disrupt the cells by sonication or other ways (French press), centrifuge for 30 min at 16000 x *g* and collect the supernatant containing the overexpressed proteins.
3. The clarified supernatant is loaded into a 1 ml affinity column (corresponding with the tag fused to the protein) previously equilibrated with buffer A.
4. In the case of His-tag, the column is washed with 7-8% of buffer B containing 0.5 M imidazole to remove unspecific binding and proteins are eluted using 40-70 % of buffer B. If other tag is used, elute the protein following the affinity column recommendations.
5. Additional gel filtration chromatography can be performed as a final step.

3.1.6 Heterodimer purification

To purify the heterodimers species, follow the initial steps described in 3.1.5 for cell disruption. Then, use the clarified supernatant for further affinity chromatography steps keeping in mind the nature of the fusion tags in the heterodimer. As an example, affinity chromatography using a 5 ml HisTrap column was performed to purify a heterodimer containing one His-tag in the L subunit. A 20-column volume gradient from 0 to 30 % of buffer B was used in order to separate individual L or S constructs from the heterodimer as can be shown in Figure 4B.

3.1.7 Autophosphorylation assay

The visualization of autophosphorylation in a dimeric HK allows identifying a *cis* or *trans* directionality in the reaction.

1. Prepare 10 μ l of the phosphorylation mixture with 4 μ M of the homo or heterodimer HK and 1 μ l of autophosphorylation buffer. Adjust to 9 μ l with water.
2. The reaction is carried out at room temperature and starts with the addition 1 μ l of the 10 X ATP cocktail which helps to add same amounts to all samples.
3. After 5-30 min, depending on the autophosphorylation capacity of the analysed HK (e.g. 10 min for HK853 and EnvZ), reactions are stopped by the addition of loading buffer containing 2x SDS in a ratio 2:1 (Sample:loading buffer). Important: boiling the samples could lead to the loss of the phosphorylated radioactive label, therefore, samples should be maintained at room temperature for 15 min before performing electrophoresis.
4. Then, load 8 μ l of the reaction samples in a 15 % SDS gel and run the gel at 200 volts until the bromophenol blue indicator marker arrive to the bottom of the gel.

5. Extract the gel and transfer it to a 3MM blotting paper and dry the gel.
6. Visualize the phosphorylated proteins by autorradioagraphy using the available equipment and analyse phosphorylation of the dimers using the appropriate software.

The *cis* autophosphorylation is confirmed by the lack of radioactive signal in the heterodimer $L^{\emptyset}/S^{\emptyset}$ and phosphorylation signal only in the S subunit of the heterodimer $L^{\emptyset}/S$. On the other hand, the *trans* autophosphorylation could be determined visualizing the heterodimer $(L^{\emptyset}/S^{\emptyset})$ where $L^{\emptyset}$ is the only phosphorylated subunit since the reaction takes place between subunits. A scheme of the heterodimers hypothetical pattern of autophosphorylation and the experimental results obtained with HK853 (*cis*) and EnvZ (*trans*) are shown in Figure 4C.

Cis or *trans* autophosphorylation has been related to the connection between helices $\alpha 1$ and $\alpha 2$ at DHP domain, a connection which is variable in sequence and length as it is observed by the 3D structures of this domain from different HKs (EnvZ, CpxA, VicK and HK853) (Figure 2). Thus, the interchange of this connection with a HK showing a distinct autophosphorylation pattern could be a useful strategy to provide clues on the interaction specificity for response regulator recognition as it has been described by Casino P. et al [10].

3.2 Visualizing the autophosphorylation reaction

To visualize the molecular basis of the autophosphorylation reaction, crystallization studies can be performed (Figure 5). The structure of a homodimeric HK performing the autophosphorylation reaction not only would provide clues for the directionality of this reaction but also would provide clues on

the reaction mechanism that can be further confirmed by a mutagenesis study. For this propose, the structures of different HKs have been solved by X-ray crystallography [10, 12].

3.2.1. HK expression and purification

1. The HK can be expressed and purified following the general affinity chromatography protocol described in 3.1.4-5 section or alternative protocols.
2. To increase the probability of crystallization, we recommend to remove the tag fused to the HK by proteolytic cleavage adding a specific protease in a protein:protease ratio typically used from 50:1 to 20:1. For certain proteases, such as Tobacco Etch Virus Protease (TEV) or Human Rhinovirus 3C Protease (3C / PreScission) addition of 0,5mM EDTA and 5mM β -mercatoethanol increases cleavage efficiency. The protein-protease mixture can be incubated at 4°C overnight and perform dialysis in buffer A at the same time to remove the competitor ligand (imidazole, glutathione, maltose...) for a further second affinity chromatography step (Figure 5A).
3. The cleaved protein was purified by a second affinity chromatography where the uncleaved protein is retained. The unretained protein is pooled and concentrate to <2 mL using Amicon centrifugal filters. A final gel filtration chromatography purification step over Superdex 200 column equilibrated with gel filtration buffer is recommended to eliminate aggregated forms of the protein.
4. Pool peak fractions and concentrate to 10-20 mg/mL using Amicon centrifugal filters.

3.2.2 Protein Crystallization, data collection and 3D structure determination.

To identify crystallization conditions vapour diffusion method using sitting drops is the most commonly method used [19]. The use of a liquid handing system (e.g.

Mosquito by ttp:labtech) that allow low volume drops (0.1- 0.2 μ l) is recommended in order to carry out more crystallization assays with the minimal volume of sample.

1. Set up crystallization assays by depositing in 96-well plates equal amounts (e.g. 0.2 μ l) of purified HK at 10-20 mg/ml and crystallization screen conditions. Reservoirs are filled with 50-75 μ l of the corresponding crystallization condition. Once the 96 screen conditions are set up, the plate is sealed with a transparent film and is incubated at an appropriate temperature, usually 21 or 4 °C (Figure 5B).
2. Screenings are monitored daily during the first week to detect crystal growth, then, can be monitored once a week to obtain a hit condition.
3. Optimize the initial crystallization conditions by varying pH, salt and precipitant concentration around the original hit conditions. Another option is the use additive screenings.
4. Once the conditions producing single crystals are found, scale up the volume of the crystallization assay (0.5-2 μ l for the drop and 0.5-1 mL for the reservoir) to produce crystals of higher size.
5. Test cryoprotectant capacity of the crystallization conditions by flash freezing this solution in liquid nitrogen and taking some X-ray diffraction snapshots. The absence of concentric rings characteristic of ice formation in the diffraction image will indicate that the solution is a good cryoprotectant. If ice-rings are observed, include cryoprotectant in the crystallization conditions and soak the crystals in this solution from 10 s to 2-3 min, and then flash freeze in liquid nitrogen (see Note 12).
6. Collect complete X-ray diffraction data sets and solve the structure by molecular replacement using as template one of the HK structures available in

the PDBs (Figure 5C). Pipelines for automated structure solution using molecular replacement are available in the CCP4 Suite [20] (such as Balbes, MrBump and MoRDa) or PHENIX Suite [21] as well as in the Auto-Rickshaw web server [22].

3.2.3 Functional characterization of autophosphorylation reaction

In the light of the structural data obtained for the autophosphorylation reaction, several potential catalytic residues for this reaction can be identified (Figure 6A). Thus, to assess the effect of these residues in HK autophosphorylation, a mutagenesis study together with *in vitro* phosphorylation assays can be performed (see Note 13).

The results of these assays can be compared with the mutagenic characterization of other HKs showing *cis* or *trans* autophosphorylation (e.g. HK853 showing *cis* and EnvZ showing *trans* as models).

1. Identify potential catalytic residues in the active site of the 3D structure by analysing the contacts of the substrate ATP (or a non-hydrolysable analogue) and the phosphoacceptor His, as well ions or water molecules to which they are coordinated. Compare the active center organization with the active center of other HKs previously characterized. It could be interesting to introduce mutations at the phosphoacceptor His as well as at the conserved acidic residue after the His, if it is present (Figure 6A). Likewise, introduce a mutation at a conserved polar residue in $\alpha 4$ which stabilizes the interaction between the two previous residues (the phosphoacceptor His and the acidic residue).

1. Site-directed mutagenesis of potential catalytic residues can be generated as described in section 3.1.2.

2. The mutants are expressed and purified as described in section 3.1.4-5.

3. Carry out autophosphorylation assays as it is described in section 3.1.7 and quantify the effect of the individual mutations for this activity. Compare the effect of these mutations with similar mutations reported in other HKs showing *cis* and *trans* autophosphorylation such as HK853 and EnvZ (Figure 6A). For HK853 and EnvZ, mutations at the conserved acidic residue completely abolished phosphorylation highlighting the relevance of this residue in the autophosphorylation mechanism (Figure 6B). Mutations at the conserved polar residue in $\alpha 4$ had a deleterious impact in the autophosphorylation demonstrating its role stabilizing the interaction between the phosphoacceptor His and the acidic residue (Figure 6B). Additional mutations at other specific residues, such as mutation at the conserved Asn in $\alpha 5$ in charge of chelating the Mg^{2+} cation, completely abolished phosphorylation due to impair binding of ATP at the active center (Figure 6B). Also, mutations in the conserved basic residues that contact the β and γ phosphates of ATP showed low phosphorylation levels mainly related with a decreased ATP binding level and/or the stabilization of the negative charge produced in the ADP product (Figure 6B). Similar results obtained for the HK of study would demonstrate that it presents a canonical autophosphorylation mechanism [10]. If differences in the effect of the mutations are observed would suggest a different mechanism of autophosphorylation and a deeper characterization of the HK would be required in order to identify molecular peculiarities.

4. Notes

Note 1. HK DNA can be obtained by PCR using as template the genomic DNA of the corresponding bacteria or from a synthetic gene of the HK. Synthetic genes

can be designed using codon optimization to enhance protein expression in *E. coli*.

Note 2. Primers should be designed such that they incorporate the overhang necessary to subclone the HK cNDA into the required plasmid using the preferred cloning strategy (e.i. LIC [23], In-Fusion HD Cloning Technology (Clontech) or traditional restriction).

Note 3. Plasmids where protein expression is under the control of an inducible promoter such as T7/lacO promoter should be used. It is necessary to use two plasmids with identical or different origins of replication but that provide resistance to different antibiotics. These plasmids should ideally allow the fusion of the HK to two different tags in order to provide short and long versions of the protein. In our study, we used as plasmids pLIC-SGC1 and pNIC28-Bsa4 [23], containing both N-terminal His-tag to produce long versions of the HK, and pET21d and pET24a (Novagene) without tag to produce short versions of the HK.

Note 4. T4 polymerase is required to create overhangs with complementarity between the plasmid and insert in the case of LIC cloning.

Note 5. It is important to choose a suitable *E. coli* strain for HK expression. It will depend on the presence of rare codons in the HK, if any toxicity is caused to the bacteria during expression and to the level of expression needed. For example, the cell strain BL21-codonplus (DE3)-RIL uses T7 RNA polymerase under the IPTG-inducible lacUV5 promoter to produce high levels of expression. This strain contains a plasmid with chloramphenicol (Clo) resistance that provides extra copies of the tRNA genes that recognize the Arg codons AGA and AGG, the isoleucine codon AUA and the Leu codon CUA.

Note 6. The pH of the buffer will depend on the pI of the protein. Addition of 1mM of phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity and 1mM of β -mercaptoethanol to avoid oxidation is recommended during lysis.

Note 7. An example of mutated residues involved in ATP binding could be N380A in HK853 and N347A in EnvZ which corresponds to a conserved Asn that comprises the N-box in the CA domain in charge of binding the Mg^{2+} ion that stabilizes de ATP. Mutation to Ala of this residue impairs binding to the Mg^{2+} ion.

Note 8. Alternatively, to the conserved Asn in the N-box, other residues could be mutated to Ala in order to avoid ATP binding and impair the kinase activity. However, these other residues should be checked for loss of ATP binding by performing ligand binding assays.

Note 9. The cytoplasmic catalytic portion of some HKs may undergo a phenomenon called spontaneous subunit exchange based on a continuously forming and breaking protein dimer. This phenomenon could affect the stability of the heterodimer formation. This is the case of the HK EnvZ that show *trans* autophosphorylation where the heterodimer $L^{\emptyset}S$ shows both subunits phosphorylated due to the formation of wild-type S dimer. In this case, it is necessary to produce the heterodimer $L^{\emptyset}S^{\emptyset}$ to confirm which subunit has become phosphorylated.

Note 10. To generate the heterodimers, the plasmids used to express the L and S versions of the protein must have resistance to different antibiotics. Thus, the inclusion of both antibiotics in the culture medium will force their maintenance in the *E. coli* strain. It is also advisable that the expression of both proteins is under the control of the same promoter and that both plasmids present a similar number of copies, thus ensuring a similar expression level of L and S versions.

Note 11. Purification of lower amounts of protein (e.g., produced in 20 ml of culture), can be performed in batch using a Ni²⁺ High Density resin (ABT) and following the indications of the supplier.

Note 12. Glycerol, sucrose or ethylene glycol, as well as high concentrations (> 30%) of polyethylene glycols (PEGs), can be good options as cryoprotectant depending on the crystallization conditions.

Note 13. The role of the catalytic residues proposed in HK853 and EnvZ was determined by their autophosphorylation capacity, but this capacity is directly dependent on the ability to bind ATP. Thus, to be able to distinguish if the contribution of a catalytic residue was solely based on the enzymatic reaction or on the ATP binding it is advisable to check the contribution of the mutation in their ATP binding capacity through Isothermal Calorimetry assays (ITC) or Surface Plasmon resonance (SPR) using AMPPNP, a non-hydrolysable ATP analogue.

Acknowledgements

This work was supported by Spanish Government (Ministry of Economy and Competitiveness) grants BIO2016-78571-P to A.M and BFU2016-78606-P to P.C. is the recipient of a Ramón y Cajal contract, from the Ministry of Economy and Competitiveness. C.M-M is the recipient of a PhD fellowship from the Programa de becas, Secretaría de Educación Superior, Ciencia, Tecnología e Innovación of Ecuador Government (2015-AR2Q9228)

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Figure captions

Figure 1. *Cis* and *trans* autophosphorylation. 3D structures of the catalytic domains of HKs showing the conformation for autophosphorylation. Two orthogonal views of EnvZ^{chim} (PDB:4k4p) showing *cis* autophosphorylation and CpxA (PDB:4cb0) showing *trans* autophosphorylation are represented in cartoon. In the dimers, each subunit is represented in a different color, green and blue for the *cis* HK subunits and orange and purple for the *trans* HK subunits. The ATP ligands are represented in stick with carbons in red. The DHp and CA domains conforming the HK catalytic domains are labelled as well the $\alpha1$ - $\alpha2$ helices and phosphoacceptor His in the DHp domains.

Figure 2. Sequence alignment of the DHp. The sequences of DHp domains from HKs with *cis*, HK853 and VicK, and *trans*, EnvZ and CpxA, autophosphorylation are aligned. The alignment was done with PRALINE webserver and the code of colours for residue conservation is in the top of the figure. The secondary structure elements of the DHp is shown at the top of the alignment. The sequence and size variable connector between $\alpha 1$ - $\alpha 2$ helices is indicated.

Figure 3. Directionality of the $\alpha1$ - $\alpha2$ DHp connection in HKs. A) Superimposition of DHp domains from HK853 (red tones; PDB: 3DGE) and Vick (blue tones; PDB: 4I5S) HKs which show *cis* autophosphorylation and from EnvZ (green tones; PDB: 4CTI) and CpxA (orange tones; PDB: 4BIU) that show *trans* autophosphorylation. B) Superimposition of DHp domains of *cis* and *trans* autophosphorylation HKs, HK853 and EnvZ respectively. At the bottom of the panel is represented an apical view of the superimposed structures showing counter-clockwise or clockwise direction of the $\alpha1$ - $\alpha2$ DHp connection.

Figure 4. Heterodimer formation. A) Schematic representation of heterodimer species labeling the subunits accordingly to the absence (L and S) or presence (L^Φ and S^Φ) of mutation is shown in the left side of the scheme. In the right side the phosphorylated subunits are coloured in each heterodimer as if the autophosphorylation reaction was carried out in *cis* (blue colour) or in *trans* (orange colour). B) Example of affinity chromatography for heterodimer purification. The chromatogram corresponds to a 5-ml HisTrap FF column eluted with a gradient of buffer B (red line) in 20 column volumes. Samples from each 3ml-fraction were loaded in a 15% SDS-PAGE gel and those fractions containing an equal ratio of L and S subunits as heterodimer (region in purple shadow) were selected. C) Visualization of heterodimer autophosphorylation. *Left*, Schematic representation of autophosphorylation patterns with samples obtained in A) in a hypothetical SDS-PAGE gel. *Right*, autoradiography of an experiment carried out with the heterodimers for HK853 (*cis*) and EnvZ (*trans*). Phosphorylation of L^Φ subunit in L^ΦS^Φ heterodimer allows to identify the *trans* autophosphorylation (in blue) while phosphorylation in S subunit in L^ΦS heterodimer allow to identify the *cis* autophosphorylation (in orange).

Figure 5. Scheme of the steps to determine the 3D structure of a HK by X-ray crystallography. A) SDS PAGE showing the purification steps of the HK before and after removal of the tag. Red and blue asterisks indicate the band corresponding to the HK with and without tag, respectively. Yellow and orange asterisks indicate the bands corresponding to the TEV polymerase and the tag, respectively, that elute with 100% of buffer B. B) MRC2 plates for protein crystal growth in an appropriate screening. C) Pictures of synchrotron (ALBA, Spain) and X-ray beamline (XALOC in ALBA) used for protein crystals diffraction. Images containing diffraction patterns are processed with computer suites such as CCP4 of Phenix to obtain the final 3D structure of the HK.

Figure 6. Residues involved in the catalytic mechanism of the autophosphorylation reaction. A) Detail of the active center in the 3D structure of EnvZ^{chim} (in green) showing the close distance between AMPPNP (in sticks with carbom and phosphate atoms in grey and orange, respectively) and the phosphoacceptor His. Superimposed DHp and CA domains of HK853 over EnvZ^{chim} structure are shown in pink. Catalytic relevant residues are drawn as sticks and labelled. B) Autophosphorylation activity assays of Ala mutants in relevant catalytic residues of HK853 and EnvZ.