doi:10.1016/j.jmb.2008.10.027 J. Mol. Biol. (2009) 385, 51–64

JMB

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The HupR Receiver Domain Crystal Structure in its Nonphospho and Inhibitory Phospho States

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Received 23 May 2008; received in revised form 30 September 2008; accepted 8 October 2008
Available online
19 October 2008

Hydrogen uptake protein regulator (HupR) is a member of the nitrogen regulatory protein C (NtrC) family of response regulators. These proteins activate transcription by RNA polymerase (RNAP) in response to a change in environment. This change is detected through the phosphorylation of their receiver domain as part of a two-component signalling pathway. HupR is an unusual member of this family as it activates transcription when unphosphorylated, and transcription is inhibited by phosphorylation. Also, HupR activates transcription through the more general σ^{70} transcription initiation factor, which does not require activation by ATPase, in contrast to other NtrC family members that utilise σ^{54}. Hence, its mode of action is expected to be different from those of the more conventional NtrC family members. We have determined the structures of the unphosphorylated N-terminal receiver domain of wild-type HupR, the mutant $HupR^{N}_{D55E}$ (which cannot be phosphorylated and down-regulated), and HupR in the presence of the phosphorylation mimic BeF_3^-. The structures show a typical response regulator fold organised as a dimer whose interface involves α4–β5–α5 elements. The interactions across the interface are slightly different between apo and phospho mimics, and these reflect a rearrangement of key conserved residues around the active site aspartate that have been implicated in domain activation in other receiver domain proteins. We also show that the wild-type HupR receiver domain forms a weak dimer in solution, which is strengthened in the presence of the phosphorylation mimic BeF_3^-. The results indicate many features similar to those that have been observed in other systems, including NtrC (where phosphorylation is activatory), and indicate that recognition properties, which allow HupR to be active in the absence of phosphorylation, lie in the transmission of phosphorylation signals through the linker region to the other domains of the protein.

Keywords: HupR; response regulator; beryllium fluoride phosphorylation mimic; X-ray crystallography

Edited by R. Huber

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Abbreviations used: HupR, hydrogen uptake protein regulator; NtrC, nitrogen regulatory protein C; RNAP, RNA polymerase; $HupR^{N}_{WT}$, N-terminal receiver domain of wild-type HupR residues 1–140; $HupR^{N}$, N-terminal receiver domain of HupR; EM, electron microscopy; PDB, Protein Data Bank; GST, glutathione *S*-transferase; SeMet, selenomethionine; MTG, monothioglycerol; ESRF, European Synchrotron Radiation Facility.

Introduction

In order to survive in changing environments, organisms need to be able to sense and adapt to variations in their surroundings. Two-component signalling systems are prokaryotic biological pathways that allow rapid sensor/response reactions to occur on timescales of milliseconds (e.g., chemotaxis[1]) to several hours (e.g., sporulation[2]). These systems also occur in some archaea, lower eukaryotes, and plants. Typically, the two-component systems comprise a sensor histidine kinase and a response regulator (reviewed by Stock *et al.*[3]). In the presence

of a signal, the histidine kinase catalyses the ATP-dependent autophosphorylation of a specific histidine residue and presents the phosphoryl group for transfer to a conserved aspartate in the N-terminal regulatory domain of the response regulator. The signal may activate or repress the response regulator's effector domain and thus regulate RNA polymerase (RNAP) activity.

Hydrogen uptake protein regulator (HupR) from the photosynthetic nonsulphur bacterium *Rhodobacter capsulatus* is a response regulator that, together with the histidine kinase HupT, forms a two-component signalling system. The signalling pathway promotes transcription of a membrane-bound H_2 uptake NiFe-hydrogenase in response to the presence of gaseous hydrogen (either exogenously produced or endogenously produced by nitrogenase activity) or low cellular organic carbon levels.[4] The membrane-bound hydrogenase transports hydrogen across the membrane and feeds electrons into the respiratory chain via cytochrome *b* (HupC), allowing the organism to grow under chemoautotrophic or photoautotrophic conditions. HupR is a 53-kDa protein consisting of three domains: (1) an N-terminal receiver domain (residues 1–140); (2) an 'AAA$^+$ ATPase-like' central domain (residues ~171–418); and (3) a C-terminal helix–turn–helix DNA-binding domain (residues ~434–480). Two linker regions join the domains. The first consists of ~40 amino acids (N-terminal to central), and the second consists of ~16 (central to C-terminal domain).

HupR belongs to the nitrogen regulatory protein C (NtrC) family of response regulator proteins based on sequence similarity and domain organisation.[5] Most NtrC family members regulate transcription from the alternative σ^{54} RNAP complex in response to receiver domain phosphorylation.[6] Transcription initiation requires an input of energy, which is provided through ATP hydrolysis[7–11]—a process that is dependent on central domain oligomerisation in response to receiver domain phosphorylation.[12–17] The phosphorylated receiver domain either promotes oligomerisation by direct binding to the central domain or relieves a previous imposed inhibition.

HupR exhibits three major differences from typical NtrC family members. Firstly, from promoter sequence analysis,[4] HupR is likely to activate transcription through the more general σ^{70} transcription initiation factor in complex with RNAP, which does not require activation by ATPase.[18] However, there is no information on how the protein interacts with RNAP. Secondly, although sequence comparison shows that HupR has an AAA$^+$ ATPase-like domain (Supplementary Material, Fig. S1), the response regulator has no ATPase activity as a result of mutations in its ATPase domain.[19] It also lacks the GAFTGA motif necessary for interaction with the σ^{54} transcription initiation factor. Interestingly, NtrC from *R. capsulatus* (NtrC_Rhoca) also lacks this motif and is also expected to utilise the σ^{70} RNAP where transcriptional activity requires ATP binding but not hydrolysis.[20] Both proteins HupR and NtrC_Rhoca possess the conserved R-finger and sensor II residues characteristic of the AAA$^+$ ATPase family (Supplementary Material). Thirdly, HupR activates transcription when unphosphorylated, and its activity is repressed on phosphorylation.[4] Activity in the absence of phosphorylation is unusual, but has been demonstrated for another H_2-sensing system, HoxA (which is similar to HupR), in *Alcaligenes eutrophus*.[21]

All response regulators contain a conserved N-terminal regulatory domain consisting of a $(\beta\alpha)_5$ fold. The activation mechanism of the NtrC family has been studied through comparisons of native and phosphoactivated forms using the BeF_3^- compound to mimic the unstable phosphoaspartate.[17,22–25] Six conserved residues have been observed at the phosphoactivation site[26] (referred to as the 'active site'): Asp12, Asp13, Asp55, Ser/Thr83, Phe/Tyr (Phe103), and Lys106, where numbers refer to the HupR sequence. Previous studies have shown that modification of the active site aspartate by phosphorylation results in a conformational change in the positions of the conserved threonine and aromatic residues. The threonine moves to contact the fluoride atom of the phospho mimic of BeF_3^-, and the aromatic group shifts from a solvent-exposed position to a buried inward position. Different response regulators respond with different outcomes, but in the NtrC family, phosphorylation results in the oligomerisation of the central domain.[12]

Here we present the structures of the unphosphorylated N-terminal receiver domain of wild-type HupR residues 1–140 ($HupR^N_{WT}$), the mutant $HupR^N_{D55E}$ (which is constitutively active and not subject to regulation by phosphorylation), and $HupR^N_{WT}$ in complex with the phosphorylation mimic BeF_3^-. The results show that the N-terminal receiver domain of HupR ($HupR^N$) responds with characteristic conformational changes observed in other response regulators on phosphorylation in both $HupR^N_{D55E}$ and $HupR^N_{WT}/BeF_3^-$ complex. We have investigated the oligomeric state of the isolated receiver domains with the wild-type protein and the mutants $HupR^N_{D55E}$, $HupR^N_{D55S}$, and $HupR^N_{D55\Delta}$ in the presence and in the absence of BeF_3^-, and we show that the phosphorylation mimic BeF_3^- promotes dimerisation.

Results

Crystal structure of $HupR^N_{WT}$

The structure of the unphosphorylated $HupR^N_{WT}$ was solved at 2.1 Å resolution (Table 1). The overall structure of $HupR^N_{WT}$ follows the classic receiver domain fold observed in all response regulator proteins. This consists of a central parallel five-stranded β-sheet (topology β2–β1–β3–β4–β5) flanked by two α-helices on one side (α1 and α5) and by three α-helices on the other side (α2, α3, and α4). The aspartate residue (Asp55) phosphorylated