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In trans regulation of the GlnD protein futile cycle of *Herbaspirillum seropedicae* by the ACT domain

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Abstract

PII are signal transduction proteins that integrate metabolic signals and transmit this information to a large number of protein targets through protein-protein interaction. The bifunctional enzyme GlnD covalently modifies PII proteins by uridylation (UTase activity) when fixed nitrogen is scarce and by deuridylyation (UR activity) under nitrogen excess conditions. Here, we characterize the *in vitro* regulation of uridylylation activity of H. seropedicae GlnD protein by truncated versions of the regulatory GlnD domains. The GlnDΔACT protein, lacking the ACT regulatory domains, catalyzes the futile cycle of PII uridylylation and deuridylylation. To attempt restoring the proper regulation of GlnDΔACT, we expressed, purified and tested the regulatory capacity of truncated versions of the ACT domains. The truncated GlnD ACT1 domain was able to control the futile cycle, indicating that we successfully developed a tool to study the regulatory mechanism of enzyme activities of GlnD.

Introduction

Nitrogen metabolism in many gram-negative bacteria is controlled by the general nitrogen regulatory (NTR) system, in which PII proteins play a key role in controlling the activity of target proteins through protein-protein interaction (1). By integrating cellular nitrogen and carbon status (2-OG levels) with energy status (ATP/ADP ratio), these proteins function by signaling intracellular levels of key metabolites (2). In addition to regulation through binding to allosteric effectors, PII can be switched between two forms by reversible covalent modification of a residue in the *T-loop* (1). Under nitrogen-limiting conditions, PII are modified by uridylylation, and under nitrogen-excess conditions, PII are deuridylylated. Both reactions (uridylyltransferase (UTase) / uridylyl removing (UR)) are catalyzed by the bifunctional enzyme GlnD (3) . This enzyme has 4 domains, of which the N-terminal domain encodes the UTase activity and the adjacent HD domain encodes the UR activity. In addition, GlnD has two C-terminal ACT domains, involved in the regulation of GlnD activity, through glutamine binding (4).

Materials and methods

To verify the activity of ACT domais of protein GlnD protein from *H. seropedicae, in silico* analysis of different ACT domain truncation points (GlnD ACT1, GlnD ACT2 and GlnD ACT3) was performed.

The gene fragment encoding the ACT domains of *H. seropedicae* GlnD was cloned into the pETM11 vector for the expression of the N-terminal His-tagged protein. The purity level of the protein was analyzed by SDS-PAGE and the protein concentration was measured by 280 nm absorbance in a Nanodrop microvolume spectrophotometer (Thermo Fischer Scientific). Uridylylation reactions were performed with GlnK (30 µM), 1 mM ATP, 0,2 mM UTP and 2 mM 2-OG, in reaction buffer (100 mM Tris-Cl pH 7.5; 100 mM KCl and 25 mM MgCl2). The bands were visualized on native PAGE and quantified to calculate the uridylylation state of the PII proteins by using ImageJ software.

Results

Through alignment analysis of the GlnD amino acid residues of *H. seropedicae*, three versions of truncated ACT domains for expression of the ACT domains of this protein were defined **(Fig. 1)** and constructed through PCR reactions **(Fig. 2)** , denominated ACT1, ACT2 ACT3.

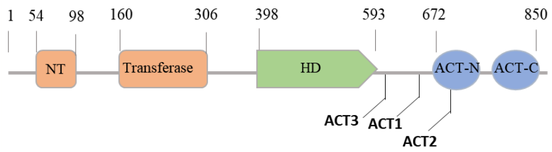

Fig. 1. Truncation points of the ACT domains of *H. seropedicae* GlnD protein

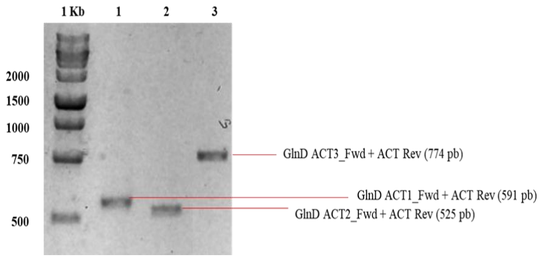

Fig. 2. Electrophoresis of *glnD* gene fragments encoding truncated ACT domains. 1. GlnD ACT1. 2. GlnD ACT2. 3. GlnD ACT3

Analysis of purifications by SDS-PAGE indicates that all proteins were purified **(Fig. 3)**

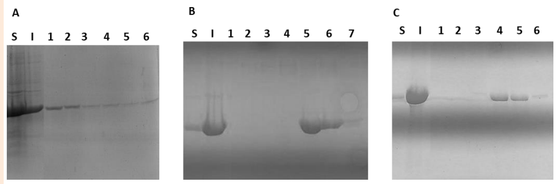

Fig. 3. Purification of A. GlnD ACT1, B. GlnD ACT2 and C. GlnD ACT3 proteins. The fractions soluble (S), insoluble (I) and the imidazole gradient are also indicated.

In order to verify the regulatory role of the ACT domains of *H. seropedicae* GlnD, we constructed a truncated version of GlnD lacking both ACT domains of its C-terminal portion, the GlnDΔACT protein (**Fig. 4A**). In addition, we added the truncated versions of the ACT domains in order to prevent *in trans* this futile cycle (**Fig. 4B-D**), and as a control, the same experiment with wild GlnD (**Fig. 4E**)

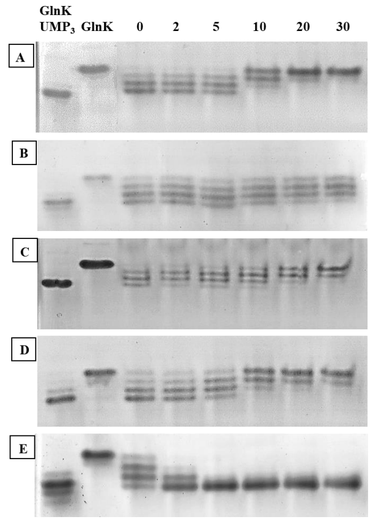

Fig. 4. Effect of UTP concentration on the uridylylation of *H. seropedicae* GlnK protein. The proteins used to catalyze the reactions were A. GlnDΔACT, B. GlnD ACT1, C. GlnD ACT2, D. GlnD ACT3 and E. wild GlnD.

Conclusion

The ACT domains of the GlnD enzyme play a regulatory role, promoting a reciprocal regulation of UTase and UR activities. Thus, the GlnDΔACT protein catalyzes a futile cycle of uridylylation/deuridylylation of the PII protein until the total consumption of the UTP substrate present in the reaction (5). Here, the ability of the truncated versions of the ACT domains to prevent *in trans* the futile cycle catalyzed by the GlnDΔACT enzyme in the presence of the 2-OG effector, as it happens in wild GlnD, was tested. The truncated ACT1 variant showed higher regulatory activity, however, ACT2 and ACT3 also appear to prevent in lesser degree the futile cycle catalyzed by GlnDΔACT. This shows that the ACT domains seem to have been able to form an intermolecular complex with GlnDΔACT to regulate its activity in response to the allosteric regulator.

Recommendations

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