Experimental report

23/01/2024

Proposal: 8-03-1077 **Council:** 4/2023

Title: Structural properties of the Caulobacter crescentus copper resistanceprotein, PcoB

Research area: Biology

This proposal is a resubmission of 8-03-1065

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Samples: D2O
Deuterated sodium dodecyl sulfate
CuSO4
deuterated n-Dodecyl ß-D-maltoside
deuterated 2-methyl-2,4-pentanediol
protein recombinante purifiée PcoB

Instrument	Requested days	Allocated days	From	To
D22	1	1	26/11/2023	27/11/2023

Abstract:

Copper cations play fundamental roles in biological systems, such as in folding and stabilizing proteins, or in enzyme reactions. While essential to the proper functioning of cells, copper in too high concentration can become toxic to them, and organisms have therefore developed tight regulatory mechanisms towards these cations. For instance, the Pco system found in the Caulobacter crescentus bacterium, is composed of two proteins: a soluble periplasmic protein PcoA and an outer membrane protein PcoB. PcoA oxidizes Cu+ to Cu2+, and PcoB is thought to be an efflux pump for these divalent cations. Whilst the PcoA protein has already been studied, much less is known about PcoB structure and function. In the present project, we plan to understand the structural properties of the native PcoB, and to validate its refolding by a peculiar method based on the association of sodium dodecyl sulfate (SDS) and 2-methylpentane-2,4-diol (MPD). First, the native PcoB extracted from the Caulobacter crescentus membrane will be studied while solubilized using the match-out deuterated N-Dodecyl-Beta-D-Maltoside. Then the refolding of the d-SDS denatured protein by adding d-MPD will be monitored.

Research proposal: Structural properties of the *Caulobacter crescentus* copper resistance protein, PcoB (8-03-1077 experiment)

Context:

Metal ions are essential components of all living cells. Among them, copper (Cu) is mainly involved in enzymatic redox reactions, as it can oxidize a wide variety of substrates by cycling between the cupric Cu(II) and cuprous Cu(I) forms. However, like many other transition metals, Cu is toxic when in excess, causing growth arrest and cell death. This feature is exploited as a bactericide in medical and biotechnological applications, but also by the mammalian immune system. As a response, many bacteria have evolved resistance to copper ions by various mechanisms like Cue, Cus or Pco systems, involved in Cu oxidation and efflux, respectively. Therefore, selectively targeting the prokaryotic copper regulation system is a fundamental stage in developing next-generation antibiotics.

The aquatic alpha-proteobacterium *Caulobacter crescentus* is a pioneer bacterial species of polluted environments because it encodes several heavy metal resistance genes. Nevertheless, the molecular mechanisms involved in this resistance process are largely unknown and differ from *E. coli* and *Pseudomonas* strategies, whose Cu homeostasis is the most studied. In *C. crescentus*, a two-component PcoAB system predominates: (i) PcoA, which has a well- described classical periplasmic multicopper oxidase activity, oxidizing Cu^{+} to Cu^{2+}, is homologous to the *E. coli* CueO system; (ii) PcoB, which is located in the outer membrane and is supposed to act as an efflux protein to selectively export Cu outside the cell. Whilst data have been published for PcoA systems, very little information is available for PcoB.

Objective: The general objective of the project is to characterize the structure and function of PcoB.

Results:

The PcoB protein is produced in the form of inclusion bodies (biologically inactive protein aggregates) and is consequently purified in a denatured form. To study the function of this protein, it is refolded using the SDS-MPD method developed in our laboratory. Initial Small-Angle Neutron Scattering (SANS) measurements are performed on both the denatured state and the presumed folded state of the protein (**Fig.1**). After obtaining the theoretical I(0) of the protein and comparing it with the experimental I(0), it appears that the protein in solution is not in its monomeric form. This holds true for both the denatured form (where the experimental I(0) is 4 times higher) and the folded form (where the experimental I(0) is 20 times higher). Subsequent SANS measurements were conducted on the same sample (10 hours after the initial measurement), with the addition of CuCl2 in a 1:1 ratio with the protein. The hypothesis is that PcoB binds to the divalent copper cations, potentially inducing conformational changes. Following the addition of copper cations to the previously folded protein, an aggregation phenomenon takes place. However, when the protein is folded in the presence of Cu^{2+}, an oligomeric state previously observed appears to be present.

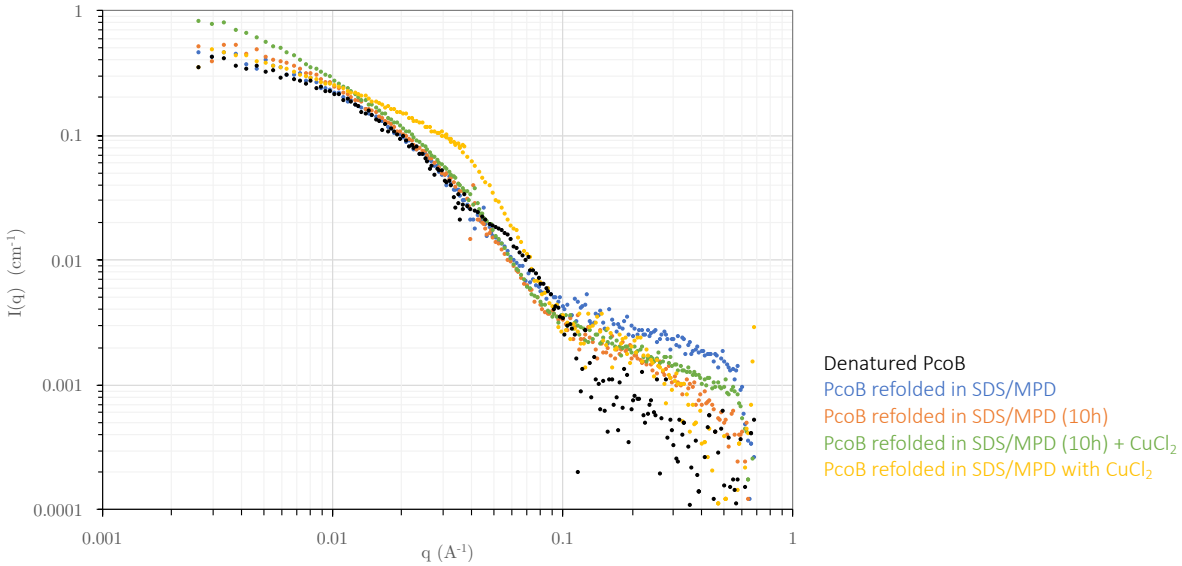

Fig.1 SANS profile of the refolding of PcoB in SDS/MPD. The buffer used consists of Tris-HCl 20 mM prepared in D_2O, and the SDS and MPD used are deuterated.

To complement our study, a mutant with the disordered N-terminal part truncated (ΔN-ter PcoB) is produced, purified, and refolded in the same way. SANS measurements are also carried out on both the denatured and folded states (**Fig.2**). Unlike what is observed for the entire protein, no plateau is reached at low q values for the mutant. This would suggest that the mutant is in the form of aggregates and could explain the lower stability observed for this protein in solution over time. Based on these results, no further tests have been conducted on this protein construct.

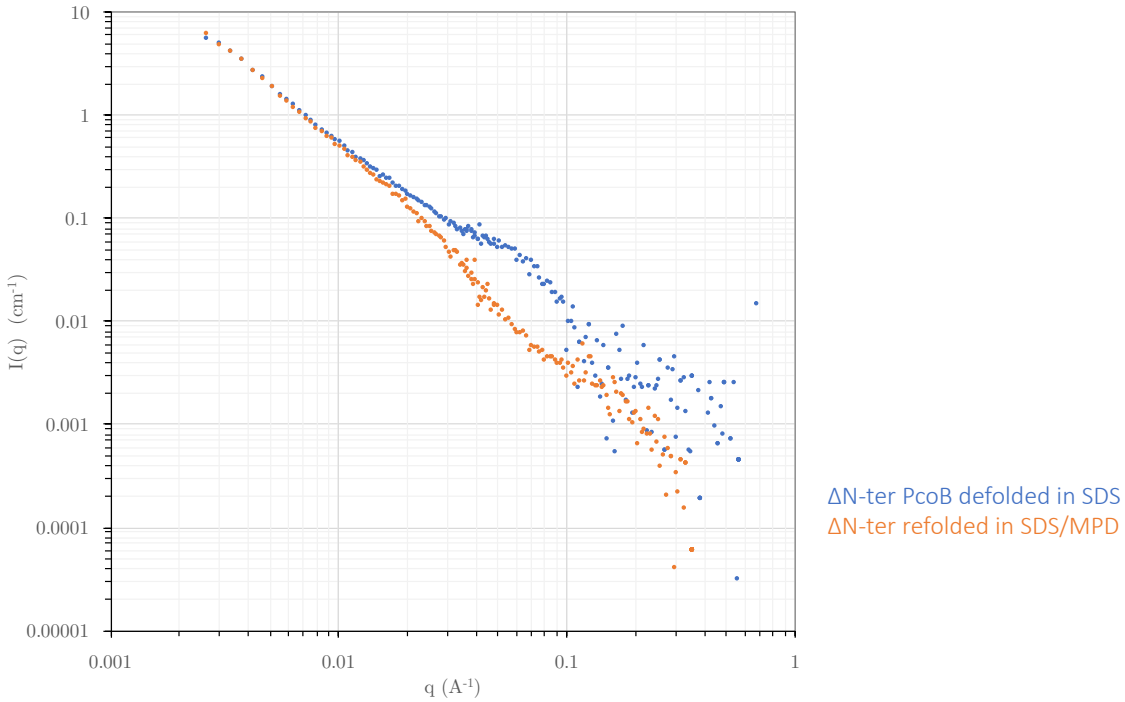

Fig.2 SANS profile of the refolding of ΔN-ter PcoB in SDS/MPD. The buffer used consists of Tris-HCl 20 mM prepared in D_2O, and the SDS and MPD used are deuterated.

In the end, another refolding method was investigated. This involved solubilizing the inclusion bodies in 8 M urea instead of the SDS detergent. Subsequently, the protein is purified

under denaturing conditions and refolded using a mild detergent known for folding membrane proteins, deuterated DDM. The folding was carried out using affinity chromatography as well as by dilution, and the samples were then analyzed by SANS (**Fig.3**). After analyzing the I(0) values, it appears that refolding by dilution does not lead to aggregation, but oligomers are present. On the other hand, the protein denatured in urea appears to be in its monomeric form, unlike the denatured form in SDS. Furthermore, another detergent (LMNG) has been investigated. This detergent is less common in the literature but is more similar to lipid characteristics than DDM. A plateau is observed for low q values. According to the I(0), the oligomers would be composed of 40 monomers.

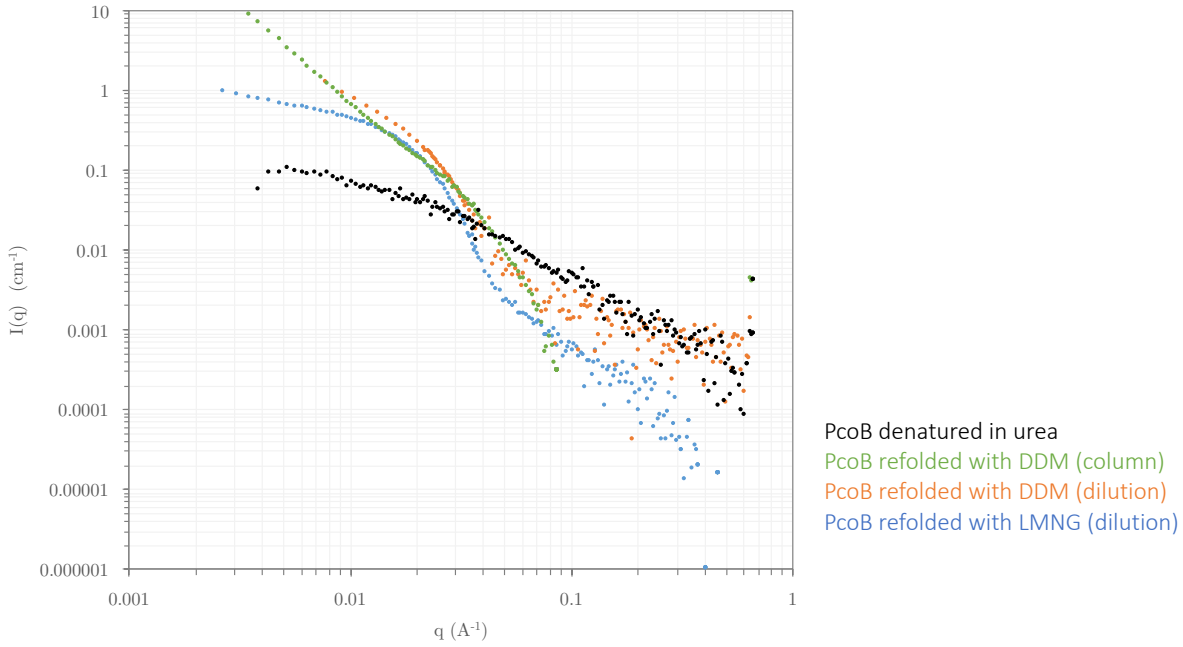

Fig.3 SANS profile of the refolding of PcoB urea/DDM and urea/LMNG. The buffer used comprises Tris-HCl prepared in D2O, and the DDM and LMNG used are deuterated.

Prospects

In future experiments, it would be interesting to analyze the protein incorporated into nanodiscs to study it in its monomeric form and understand the potential effects of copper cations on the disordered chain (disorder to order transition?). Additionally, it would also be worthwhile to test various conditions to prevent the aggregation of both the protein and the mutant (salt concentration, pH, temperature).