NMR localization of the O-mycoloylation on PorH, a channel forming peptide from Corynebacterium glutamicum

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PorH and PorA are two small peptides that, in complex, form a voltage-dependent ion channel in the outer membrane of Corynebacterium glutamicum. Specific post-translational modifications on PorA and PorH are required for the formation of a functional ion channel. The assignment of PorH proton NMR chemical shifts in DMSO, allowed identifying unambiguously the exact position of the PorH O-mycoloylation on Ser 56 side chain. This was further confirmed by site directed mutagenesis and mass spectrometry. Together with the previously published localization of PorA mycoloylation, this provides the complete primary structure characterization of this outer membrane porin.

1. Introduction

Corynebacterium glutamicum, a widely used micro-organism for the production of amino-acids in industry, is an important member of the Corynebacteriales order, which also includes many infectious agents such as the etiological agents of tuberculosis and leprosy. Members of this group are Gram-positive bacteria typified by a unique cell envelope that contains an outer membrane, also called mycomembrane, involved in a permeability barrier resembling that of Gram-negative bacteria. In the case of Corynebacteria this outer membrane consists of a bilayer composed of mycolic acids, α-branched β-hydroxylated very-long chain fatty acids (C30–C36), covalently linked to the cell wall arabino-galactan, which in turn is attached to peptidoglycan, and of various non-covalently linked lipids that also include mycolic acid-containing glycoconjugates such as trehalose mono- and di-mycolate [1,2]. In addition, defined proteins, including pore-forming proteins (Por), composed the mycomembrane [3]. In C. glutamicum, we recently showed that two Por, namely PorA and PorH, are O-acylated by a mycoloyl residue, a novel form of mycolate-containing substance, which represents the first O-linked acylation onto an hydroxylated amino-acid of bacterial polypeptides [4]. We further demonstrated that this modification is critical for the pore-forming activity of the PorA/PorH hetero-oligomer [5].

While PorA mycoloylation site has been identified by point mutation and mass spectrometry, this strategy was not suitable in case of PorH due to the very large number of potential acylation sites (Fig. 1D). In the present article we present an original strategy based on the expression and purification of the 15N labelled polypeptide. By performing 2D and 3D NMR in a DMSO solution, we assigned the peptide and mycolic acid resonances, and found convincing NMR evidences (chemical shift variations and NOEs) of the mycoloylation of Ser 56. This was finally confirmed by mutagenesis and mass spectrometry.

2. Materials and methods

2.1. Chemicals

Solvents for NMR spectroscopy and 15N-NH4Cl were purchased by Euriso-top, Inc. Bacto Brain Heart Infusion was from Becton, Dickinson and company. All other chemicals were of analytical grade from Sigma.

2.2. Mutagenesis and construction used for PorH

Ponctual mutations of Serine 56 and both Ser56-Ser57 to Alanine were realized by PCR using respectively the oligonucleotides:

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were amplified in (0.2 g/l), were 15N, (0.1 M), Protocatechuic acid (30 mg/l), Biotin (200 mg/l). 15N-PorHCHis precipitate was solubilized in respectively considering different growth kinetics of l. For over-expression, cells were induced for 12 h with 1 mM IPTG. Both media contain chloramphenicol at a concentration of 10 mg/l. Further various concentrations of NH4Cl in each media. Further various concentrations of NH4Cl (from 2 to 20 g/l) and glucose (from 5 to 40 g/l) were used to improve the total cell mass and thereby the level of PorH expression. In addition, a 5 l volume fermenter (Infors, AG, Switzerland) was used to maintain the pH (7.3), temperature (30 °C), and aeration (300 rpm) of the culture, which are the essential parameters to be monitored during C. glutamicum growth, as was described previously [6–8].

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Over-expressed cells from 3 l culture were harvested by centrifugation at 4000 × g for 15 min at 4 °C followed by a washing step with 500 ml of 50 mM Tris–Cl, 200 mM NaCl, pH 8.0 (Buffer A). The washed cell pellets were weighed and thoroughly mixed with buffer A containing 0.46% LDAO (buffer B) (fivefold volume per gram of cell pellet) followed by continuous shaking at room temperature for a minimum of 3 h. The extracted cells were centrifuged twice: first, at 4000 × g for 15 min and then at 18000 × g for 30 min. The supernatant containing total cell wall proteins was passed through 3 ml of Ni-NTA affinity column (Qiagen) pre-washed with buffer B. After complete loading, the immobilized proteins were washed with buffer B containing 10 mM imidazole. A gradient (2–100%) of 30 ml total volume was applied to elute the proteins in buffer B containing 500 mM imidazole. The purity and homogeneity of eluted protein fractions were analyzed on 16% Tris–Tricine SDS–PAGE [9]. The fractions containing pure 15N-PorHCHis were concentrated to 2 ml using a 3 kDa cut-off Vivaspin concentrator and dialyzed against buffer B to eliminate imidazole completely.

To exchange the protein environment from LDAO to deuterated-DMSO, the dialyzed protein was precipitated in cold ethanol (20 times volume) plus 12% of 5 M NaCl water solution, and left 24 h at −20 °C. The protein precipitates were recovered by centrifugation at 10000 × g for 30 min at 4 °C. The precipitate was further dried at room temperature for 2 h to eliminate traces of ethanol. Finally the 15N-PorHCHis precipitate was solubilized in 600 µl of deuterated-DMSO at 0.95 mM peptide concentration and transferred to a 5 mm NMR tube.

2.5. NMR spectroscopy

Two dimensional 13N,1H HSQC and three dimensional HSQC-TOCSY (mixing time 80 ms) and HSQC-NOESY (mixing time 120 ms) experiments [10] were acquired at a proton Larmor frequency of 600 MHz in a Bruker Avance NMR spectrometer equipped with an HCN cryoprobe. The data were acquired at 298 K. 1H Chemical shifts were referenced to TMS using DMSO methyl resonance as internal reference (2.54 ppm). 13N chemical
shifts were referenced to $^1$H chemical shifts using a frequency ratio of 0.10132912. The spectra were processed in Topspin 3.1 and analysed in CARA [11] for the proton chemical shift assignment of PorH$_{\text{Chis}}$.

2.6. Mass spectrometric analysis

MALDI-TOF (matrix-assisted laser-desorption/ionization-time-of-flight) spectra of expressed and purified WT- and S56A-PorH$_{\text{Chis}}$ were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) fitted with a pulsed nitrogen LASER emitting at 337 nm and all spectra were analyzed in linear mode using an extraction delay of 100 ns with accelerating voltage of 25 kV. Protein samples were mixed with sinapinic acid as matrix and were loaded onto a metal plate, a total of 2500 shots were accumulated for each sample in a positive ion mode and all data were acquired with default calibration for the instrument.

3. Results and discussion

Protein post-translational modifications (PTM) are known to play crucial physiological functions and to control fundamental biological processes. The most common post-translational modifications on proteins are glycosylation, phosphorylation, acetylation, ubiquitination, cysteine oxidation and nitrosylation [12–20].

Using mass spectrometry and alkaline hydrolysis, we have previously shown that PorA and PorH from C. glutamicum are both post-translationally modified by an O-mycoloylation. In the case of PorA, which contains a limited number of serine and threonine residues, the exact position of the modification (Ser 15) could be determined by a combination of Edman degradation and site-specific mutagenesis, followed by mass spectrometry [4]. It should be noted that for both PorA and PorH, our attempts by tandem mass spectrometry failed to reveal the modified amino acids, presumably because the mycoloylated peptides were too hydrophobic to give good MS spectra. PorH possesses a large number of potential acylation sites: 1 Tyr, 5 Ser and 6 Thr (Fig. 1D). Sequence alignment of PorH with its orthologs from Corynebacterium diphtheriae and Corynebacterium efficiens showed that Thr-15 and Thr-48 residues are conserved, and we thus mutated these two residues, but the resulting polypeptides were still mycoloylated [4]. Instead of embarking in the point mutation of these 12 potential residues, we decided to use a completely different strategy based on NMR.

Indeed, NMR is a method of choice for the analysis of post-translational modifications as was recently reviewed [21]. Most classical modifications can be followed precisely, in a time resolved and quantitative manner and if desired in cellulo, thus giving a precise view of the functional dynamics of protein modifications in vivo. Theillet et al. provided a comprehensive review of protein phosphorylation, alkalyation, N- and O-glycosylation and Lysine N-acylations, and their fingerprints in NMR spectra. Protein O-acylation has been less frequently described in the literature and only a few such proteins have been described so far [22], which include the growth hormone releasing peptide Ghrelin [23] and WntA, a protein that plays a key role in numerous aspects of embryogenesis [24]. PorA and PorH were actually the first proteins described to be O-acylated in bacteria [4]. The case of Ghrelin is interesting because of the influence of its O-acylation on its proton NMR spectrum has been described with great precision [25]. Ghrelin is octanoylated on its Ser 3 hydroxyl: the most significant consequence of the acylation is an increase of the Ser H$_a$ chemical shifts by 0.46 ppm, followed by +0.31 ppm on Ser H$_x$ and +0.18 ppm on Ser N$_H$, while the effect on other amino acids are negligible [25]. Since Ser H$_a$ protons are less sensitive to primary sequence variations than NH and H$_x$ proton, they are the marker of choice to identify O-acylation of serine residues. Considering the size of PorH construct (74 aa, see Fig. 1D), we decided to make it uniformly $^{15}$N labeled and apply a standard assignment strategy based on HSQC-TOCSY and HSQC-NOESY.

PorH was produced from recombinant C. glutamicum cells grown in a minimum medium as described in material and methods. We screened various concentrations of NH$_4$Cl and glucose and observed that the concentration of glucose significantly affects the growth of C. glutamicum cells with substantial quantities of cells obtained beyond 1% of glucose while NH$_4$Cl has a marginal effect (Fig. 1). It may be noted that optimum growth of C. glutamicum requires more than 20 g/l of glucose which may be a problem for uniform $^{13}$C/$^{15}$N labelling (to be compared with 2 g/l typically used for E. coli based expression). Careful pH control in a fermenter was required since acidification of the growth medium led to a drastic reduction of PorH expression yield. The proteins extraction and purification was performed according to published procedures [5] and as described in the material and methods section, and was based on LDAO solubilisation of the cell walls. Since we had shown that cell-free expressed PorH can be solubilised and give nice HSQC NMR spectra in LDAO detergent micelles [5], we first tried to assign PorH$_{\text{Chis}}$ in this environment. However detailed inspection of the spectra rapidly revealed strong spectral heterogeneity, which could arise from either a chemical heterogeneity (due to PTM on several residues) or from conformational heterogeneity, due to slow exchange between various conformers at the micelle interface. We could demonstrate that the second hypothesis was the right one by transferring the protein into DMSO solution, where we then observed nice and unique cross peaks in the HSQC spectrum (Fig. 2A).

A series of 2D and 3D NMR spectra was acquired in order to perform $^1$H and $^{15}$N protein assignment using $^{15}$N based standard assignment strategy [26], as well as partial $^{13}$C assignment of protein residues and mycolic acid protons and carbons: NOEY, TOCSY, $^{15}$N-HSQC, $^{13}$N-HSQC-NOEY, $^{15}$N-HSQC-TOCSY, $^{13}$C-HSQC and $^{13}$C-HMBC. Nearly complete $^1$H and $^{15}$N assignment has been obtained for non proline residues (from D2 to H69) and deposited in the Biological Magnetic Resonance Data Bank under accession number 19181. It should be noted that the BMRB database is extremely poor in protein assignments in DMSO (a search on DSMO as a solvent gives 70 entries out of 8665), and that this information is useful in itself, for instance for the assessment of random coil chemical shifts and chemical shifts prediction software, as was discussed [27]. Fig. 2A shows the assignment of $^{15}$N- $^1$H amide cross peaks. Fig. 2B illustrates the outcome of the HSQC-TOCSY spectrum. Strips corresponding to several Serine residues are shown and display their Hx and Hj chemical shifts. The Serine chemical shifts (Table 1) clearly indicate that S56 present downfield shifts of +0.6 ppm for the Hj proton and of +0.2 ppm for the Hx protons as expected for an O-acylation [25]. Accordingly $^{13}$C chemical shifts display variations expected for O-acylated alcohols, i.e., +1.5 ppm in alpha position of the OH (Cj carbon) and --4 ppm in beta position of the OH (Cz carbon) [28]. The NH chemical shift at 8.63 ppm is also shifted compared to the other serine residues, except for Ser4 NH at 8.78 ppm. The other potentially acylated residues, Thr and Tyr, did not display any abnormal chemical shift.

Other NMR indications for the localisation of the mycoloylation were observed. After assigning several resonances characteristic of the mycolic acid itself (Fig. 4B), NOEs contacts were observed between proton H3 at 3.606 ppm and several Phe 55 resonances such as Hj, and Har as shown in Fig. 4A. Similarly, H2 at 2.358 ppm gave a NOE contact with Phe 55 Hj. Other NOEs could not be assigned unambiguously due to severe overlap. Due to signal to noise limitation, the HMBC failed to provide connectivity from both sides of the ester bond and CO assignment.
Although convincing evidences of the mycoloylation of Ser 56 were obtained from the NMR spectra, the absolute confirmation was finally obtained from mutagenesis and mass spectrometry. A S56A-PorHCHis mutant of was prepared by overlapping PCR, over-expressed and purified using the same procedure as for the wild type PorHCHis. The absence of mycolic acid on this mutant was verified by MALDI-TOF mass spectrometry. The theoretical mass of wild type PorHCHis is, for (M+H)+, 8677 ±1 Da with a C34:2 mycolic acid modification. As shown in Fig. 3, the S56A mutant protein has a (M+H)+ of 8158 ±1 Da, which confirms the lack of mycolic acid. The double mutant S56AS57A-PorHCHis was also produced and its mass spectrum also confirmed that it was not mycoloylated.

In conclusion, the site of mycoloylation of PorH of C. glutamicum was established by 2D and 3D NMR through 1H and 15N protein assignment and partial 13C assignment of protein residues and mycolic acid protons and carbons. It was subsequently confirmed by point mutation and mass spectrometry. PorH represents a part of the major hetero-oligomeric ion channel complex, playing a crucial role in passage of ions across the bacterial cell wall. This post-translational modification has been previously shown to affect the channel properties such as multiple conductance and slow-fast kinetics as shown by black lipid ion channel conductance measurements. The O-mycoloylation may also affect intracellular trafficking and the proper transport to the outer membrane and the assembly of a functional oligomer in vivo. The present result, together with the previously published mycoloylation site of PorA.

Table 1

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<th>Hα</th>
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provide a complete characterisation of the PorA/PorH porin at the primary structure level. This opens the way for the determination of its functional quaternary structure in a purified and reconstituted form [5], or in its native membrane environment [29].

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