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ARTICLE

NMR-assignments of a cytosolic domain of the C-terminus of polycystin-2

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Abstract Mutations in the *PKD2* gene lead to the development of polycystic kidney disease (PKD). The *PKD2* gene codes for polycystin-2, a cation channel with unknown function. The cytoplasmic, C-terminal domain interacts with a large number of proteins including mDia1, α-actinin, PIGEA-14, troponin, and tropomyosin. The C-terminal fragment polycystin-2 (680–796) consisting of 117 amino acids contains a putative calcium binding EF-hand. It was produced in *Escherichia coli* and enriched uniformly with ^{13}C and ^{15}N. The backbone and side chain resonances were assigned by multidimensional NMR methods, the obtained chemical shifts are typical for a partially folded protein. The chemical shifts obtained are in line with the existence of two paired helix-loop-helix (HLH) motifs.

Keywords Polycystin-2 · PKD2 · Polycystic kidney disease · NMR structure · EF-hand

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Introduction

Polycystin-2 is a 968-amino acid long protein that is encoded by the *PKD2* gene. The *PKD2* gene is one of the two genes mutated in patients with autosomal-dominant polycystic kidney disease (Mochizuki et al. 1996). According to structural predictions, polycystin-2 consists of 6 membrane-spanning domains; both its N- and C-terminus extend into the cytoplasm. By sequence comparison a coiled-coil domain and a Ca^{2+}-binding EF-hand have been predicted in the C-terminus of polycystin-2 but so far only indirect evidence for the presence of these motifs has been presented (Celic et al. 2008). Electrophysiological measurements by several groups have demonstrated that polycystin-2 is a non-selective cation channel and that its channel activity is regulated by calcium. The fragment of polycystin-2 studied here contains a Ca^{2+}-binding motif predicted from the amino acid sequence.

Materials

A fragment coding for amino acids 680–796 of human polycystin-2 was amplified from the PKD2 cDNA (kind gift from Steve Somlo) by polymerase chain reaction (forward primer 5′-ATTTGCCAATTGGTATCAATGAT ACTTACTCT-3′; reverse primer 5′-ATTTGCCTCGAG TCATAAAGAACTGTGATCCAA-3′) and subcloned into a modified form of the prokaryotic expression plasmid pET41a (Novagen). The modified plasmid coded for a recombinant protein with 6 histidines at the N-terminus followed by a thrombin cleavage site. The protein was overexpressed in BL21(DE3) cells (Novagen) grown in an adapted NMM medium as described by Gronwald et al. (2001). 1 L medium contained 1 g NH_4Cl, 6 g glucose,

9.4 g $Na_2HPO_4 \cdot 2\ H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 0.25 g $MgSO_4 \cdot 7\ H_2O$, 14 mg $CaCl_2 \cdot 2\ H_2O$ and 30 mg kanamycine. Finally, 5 mg EDTA-$Na_2 \cdot 2H_2O$ and 2 mg $FeSO_4 \cdot 7\ H_2O$ freshly dissolved in 900 µl H_2O and 100 µg $ZnSO_4 \cdot 7\ H_2O$, 30 µg $MnCl_2$, 300 µg H_3BO_3, 200 µg $CoCl_2 \cdot 6\ H_2O$, 10 µg $CuCl_2 \cdot 6\ H_2O$, 20 µg $NiCl_2 \cdot 6\ H_2O$ and 30 µg $Na_2MoO_4 \cdot 2\ H_2O$ dissolved in 1 ml H_2O were added. Heavy metal stock solutions were filter-sterilized before being added to the autoclaved medium. For induction of protein synthesis, cells were grown at 310 K in minimal medium to an $OD_{595} = 0.5$ and incubated 4 hours at 303 K in the presence of 1 mM isopropyl-β-D-thiogalactoside. Cells were harvested by centrifugation (4,000 rpm), resuspended in 20 mM Tris–HCl pH 8.0, 5 mM imidazole, 500 mM NaCl, 0.1% Triton-X-100 and lysed by ultrasonication (Vibra Cell, Sonics and Materials Inc.). The recombinant protein was purified over a Ni^{2+}-column (Novagen) and then dialyzed against 20 mM Tris–HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$. For labeling with ^{15}N, NH_4Cl was replaced by $^{15}NH_4Cl$ (>99%, Spectra Stable Isotopes), for double-labeling glucose and NH_4Cl were replaced by $^{13}C_6$-glucose (>99%, Spectra Stable Isotopes) and $^{15}NH_4Cl$ (see above). In case of ^{2}H, ^{15}N and ^{13}C-enrichment the same medium was used as for double-labeling, but H_2O was replaced by D_2O (>99% ^{2}H, Spectra Stable Isotopes). After dialysis, 100 µg of protein were combined with 0.3 U of biotinylated thrombin (Novagen: 1 U per µl) in a total volume of 500 µl and incubated over night at 310 K. The next morning, 32 µl of streptavidin agarose beads (Novagen: 50% suspension) were added per 1 U of biotinylated thrombin. To remove the streptavidin beads together with the biotinylated thrombin, the suspension was centrifuged (4,500 rpm) and the supernatant was loaded on a Ni^{2+}-column to remove the histidine-tail. Since the majority of polycystin-2 tended to stick to the Ni^{2+}-column, most of the protein had to be eluted with 100 mM imidazole. Finally the eluted protein and the flow through were combined and dialyzed against 10 mM K_2HPO_4/KH_2PO_4 pH 6.8, 500 mM NaCl or 5 mM Tris–HCl pH 6.8, 500 mM NaCl. The obtained polycystin-2 (680–796) fragment contains six additional amino acids (Gly-Ser-Thr-Ala-Ile-Gly) encoded by the vector.

NMR spectroscopy

Data were recorded at a magnetic field strength of 14.1 T and 18.8 T using a Bruker AVANCE 600 and AVANCE 800 spectrometer equipped with TXI and TCI cryogenic probes operating at ^{1}H resonance frequencies of 600.13 and 800.2 MHz. All NMR spectra were acquired at 293 K. In general, the NMR measurements were performed on a 0.5 mM protein sample in 10 mM potassium phosphate buffer pH 6.8, 500 mM NaCl, 2 mM DTE and 0.1 mM DSS containing 90% H_2O and 10% D_2O. ^{1}H chemical shifts were referenced to DSS used as internal standard. ^{15}N and ^{13}C chemical shifts were indirectly referenced to DSS as described by Wishart et al. (1995). When necessary, chemical shifts were corrected for the TROSY effect.

Sensitivity-improved ^{1}H-^{15}N-HSQC experiments were recorded with 128 × 2,048 complex data points using a sweep width of 8,400 Hz in the ^{1}H dimension and 2,400 Hz in the ^{15}N dimension. The sequential backbone assignment was performed on the $^{13}C/^{15}N$ labelled protein sample of polycystin-2 (680–796) using HNCA, CBCA (CO)NH, HNCACB, HNCANNH, HNCO, ^{15}N-TOCSY-HSQC and ^{1}H-^{15}N-NOESY-HSQC 3D spectra. Side chain assignments are based on HCCH-TOCSY, ^{15}N-TOCSY HSQC and 2D NOESY spectra. In some cases the TROSY versions (Pervushin et al. 1997) of the experiments were used additionally. All NMR spectra were processed and analyzed using the TOPSPIN 2.1 software (Bruker Biospin) and AUREMOL (www.auremol.de; Gronwald and Kalbitzer 2004).

Extent of resonance assignments

In the absence of $CaCl_2$ the obtained ^{1}H, ^{15}N-HSQC spectrum was poorly resolved and contained not the expected number of 123 amide back bone signals (data not shown). After addition of 5 mM $CaCl_2$ the spectral quality improved dramatically (Fig. 1). The spectrum showed satisfactory chemical shift dispersion, although still a rather large number of resonances were found at random-coil positions.

The N-terminal amino acids with the sequence GSTAIG originating from the vector show very narrow lines and typical random coil chemical shifts. Amino acids 680–706 from polycystin-2 are characterized by somewhat broader lines and chemical shift values that are still close to the random-coil values but significantly different. The amide signals of amino acids 706–724 and 784–789 are broadened beyond detection probably by slow or intermediate exchange processes. The following amino acids show large deviations from the random-coil chemical shifts and only the last 6 amino acids show chemical shifts that are again close to those expected for an unstructured peptide. As an example, the deviation $\Delta\delta(C^{\alpha})$ of the C^{α} chemical shifts from the random-coil values given by Schwarzinger et al. (2000) are depicted in Fig. 2. A positive value of $\Delta\delta(C^{\alpha})$ is correlated with a preference for α-helices, a negative value with β-structures. The application of several secondary structure predictors delivers a consistent number of helical regions. Jpred3 (Cole et al. 2008) predicts α-helical regions for amino acids 683–691, 697–715, 721–731, 739–747,

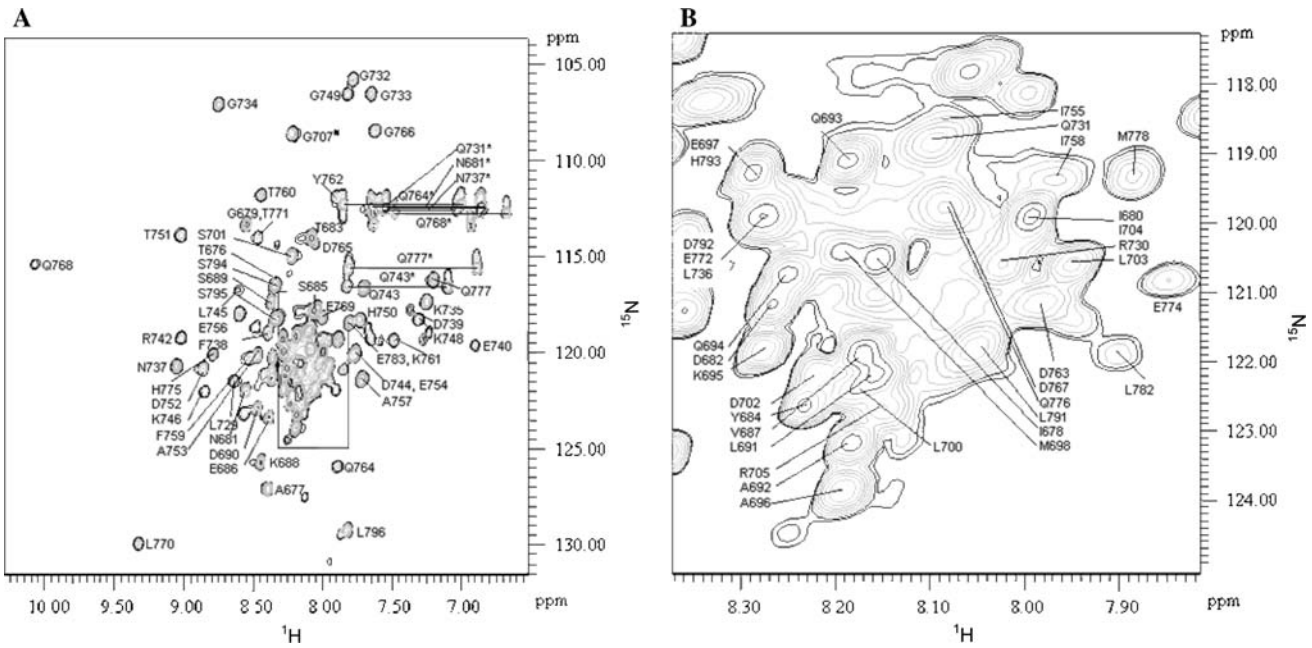

Fig. 1 ^{1}H-^{15}N-HSQC spectra of polycystin-2 (680–796). 800 MHz ^{1}H, ^{15}N-HSQC spectrum of polycystin-2 (680–796) at a temperature of 293 K. The sample contained 0.5 mM polycystin-2 (680–796) in 10 mM potassium phosphate buffer pH 6.8, 5 mM $CaCl_2$, 500 mM NaCl, 2 mM DTE and 0.1 mM DSS, 90% H_2O and 10% D_2O. **a** Full ^{1}H, ^{15}N-HSQC spectrum of polycystin-2 (680–796); **b** Sub region of the ^{1}H, ^{15}N- HSQC spectrum (marked in **a**) shown

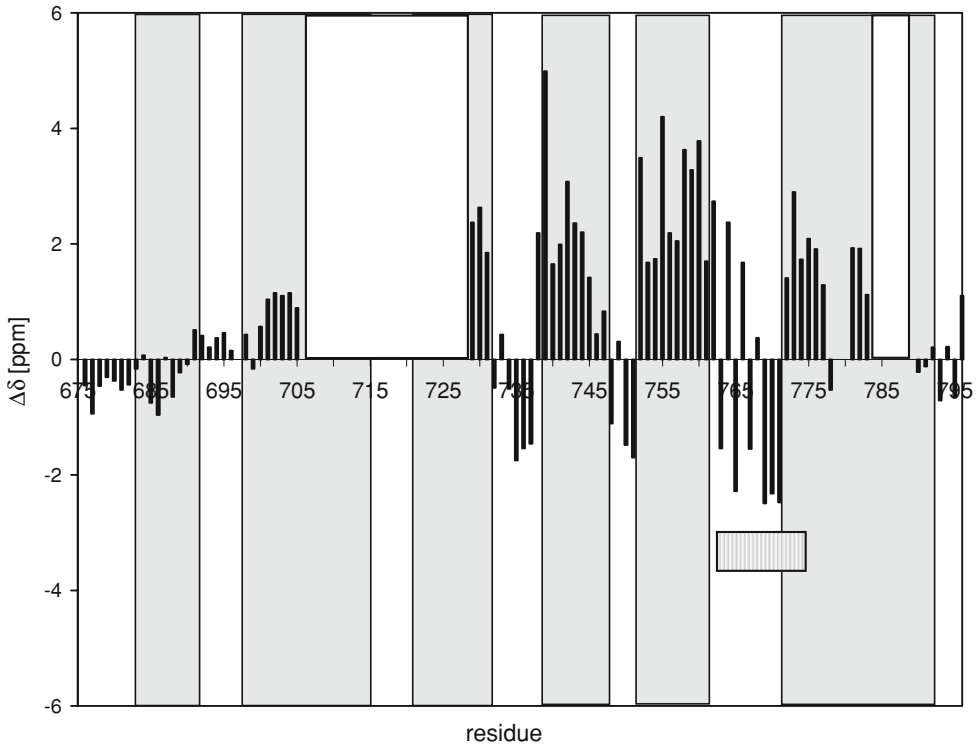

Fig. 2 C^{α}-plot and secondary prediction. Deviation of the $\Delta\delta(C^{\alpha})$ chemical shifts from the random coil values (Schwarzinger et al. 2000). Secondary structure prediction was performed with Jpred3 (Cole et al. 2008), only helical regions were predicted (highlighted *light grey*). The region for a predicted EF hand motif (D763–E774) is marked with a *grey bar*. Residues within the two *white boxes* (amino acids 706–728 and 784–789) are not visible in the heteronuclear NMR spectra

752–761 and 772–792 (Fig. 2). A sequence comparison predicts that polycystin-2 contains an EF-hand that binds calcium ions (Celic et al. 2008). Typical EF-hands consist of a helix-loop-helix (HLH) motif with two helices (E-helix and F-helix) flanking a 12 residue metal-binding loop with a typical metal binding sequence motif (Strynadka and James 1989). In fact, the predicted calcium binding loop (Fig. 2) is flanked by two regions with C^{α}-chemical shifts typical for α-helices. Typical calcium binding HLH motifs occur as closely linked pairs. Although structure prediction programs do not predict a second calcium binding site (Cai et al. 1999), C^{α}-chemical shift would agree with the existence of a second HLH motif.

Excluding the regions from amino acid 706–728 and 784–789 that contain the residues with exchange broadened amide resonances and thus cannot be observed by heteronuclear NMR-spectroscopy, approx. 95 % of all H^{N}, H^{α}, N, C′, C^{α}, H^{β} and C^{β} resonances are assigned. In addition, approx. 80 % of all other side chain resonances of the NMR-visible residues could be identified. The assignments were deposited in the BMRB data base under the accession number 16191.

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