Solution structure of pleckstrin homology domain of dynamin by heteronuclear NMR spectroscopy

DAVID FUSHMAN*, SEAN CAHILL*, MARK A. LEMMON†, JOSEPH SCHLESSINGER‡, AND DAVID COWBURN*†

*The Rockefeller University, 1230 York Avenue, New York, NY 10021; and †Department of Pharmacology, New York University Medical Center, New York, NY 10016

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ABSTRACT The pleckstrin homology (PH) domain is a recognition motif thought to be involved in signal-transduction pathways controlled by a variety of cytoplasmic proteins. Assignments of nearly all 1H, 13C, and 15N resonances of the PH domain from dynamin have been obtained from homonuclear and heteronuclear NMR experiments. The secondary structure has been elucidated from the pattern of nuclear Overhauser enhancements, from 13C chemical shift deviations, and from observation of slowly exchanging amide hydrogens. The secondary structure contains one α-helix and eight β-strands, seven of which are arranged in two contiguous, antiparallel β-sheets. The structure is monomeric, in contrast to the well-defined intimate dimerization of the crystal structure of this molecule. Residues possibly involved in ligand binding are in apparently flexible loops. Steady-state 15N(1H) nuclear Overhauser effect measurements indicate unequivocally the boundaries of this PH domain, and the structured portion of the domain appears to be more extended to the C terminus than previously suggested for other PH domains.

The pleckstrin homology (PH) domain is a modular protein of ~110 amino acids (1) that has been suggested to bind with affinity to certain lipids (2, 3), or possibly to other phosphorylated ligands including By subunits of guanine nucleotide-binding proteins (G proteins) and protein kinase C (4). Interactions between PH domains and their ligands are thought to mediate the formation of complexes involved in intracellular signal transduction, although well-characterized instances are as yet unknown.

From the sequence analysis of 3BP2, a Src homology 3 binding protein, Mayer et al. (1) have shown that one domain of 3BP2 contains a section with considerable sequence homology to several other proteins. Such a homology is suggestive that this domain may play a similar role to that of Src homology regions 2 and 3 in recognizing different protein areas in signal transduction (5). A very large number of possible PH domains have now been identified, but their comparative similarity scores are quite low, and a definitive class of absolutely conserved sequences is still hard to define (6). The solution structures of the PH of spectrin (7) and of the N terminus of pleckstrin itself (2) have been reported, as has the crystal structure (8) of the target of this paper, the PH domain of dynamin.

One mutation in a putative PH domain has been linked to genetic immunodeficiency (9). A hypothetical suggestion is that PH domains (or a subclass) are associated with cell surface receptors not containing kinases and act as cofactors or modifiers to recruited enzymic activities. Two clear examples of such are the β-adrenergic receptor kinase (reviewed in ref. 10) and the insulin receptor substrate 1 (reviewed in ref. 11). It has been suggested that this may occur specifically to G proteins (6), but there is no evidence to rule out direct PH interactions in, for example, insulin receptor substrate 1 binding to the insulin receptor or 4PS binding to the interleukin 4 receptor (11). The similarity of the structure of the PH N domain of pleckstrin to that of retinol binding protein (2) has led to the suggestion that lipids may be the natural ligands and the observation that PH domains bind to lipid vesicles, especially those containing phosphatidylinositol 4,5-bisphosphate (PIP2) (3). The lack of a large set of mutants of PH domains with defined pathology may reflect the essential role of PH domains, rather than the opposite.

Dynamin is a 100-kDa GTPase involved in the initial stages of endocytosis. In vitro, dynamin binds to microtubules, which stimulate its activity. Binding of full-length dynamin to Src homology 3 domains of proteins involved in signal transduction has also recently been reported in vitro (12-14). Dynamin family members appear to be associated with many mechanical features of differentiation, the Golgi, and cell division (15-19) and, in association with kinesin and dynein, are associated with microtubules.

In this paper, the assignments and structure of dynamin PH domain is derived from nuclear Overhauser effects (NOEs), amide exchange data, and chemical shifts obtained by a variety of homonuclear and heteronuclear NMR methods. In light of the binding (3) and sequence homology data, the structure reveals part of a putative ligand binding site and conserved structural features.

MATERIALS AND METHODS

Sample Preparation. Recombinant dynamin PH domain was obtained by expression of a pET vector product as described (8, 20). The sequence of the 125-residue protein is shown in Fig. 1. The first residue is not from the natural sequence. Uniform 13N labeling was achieved by growing the cells in minimal medium with 18 mM 15NH4Cl. Selective 15N labeling for methionine and lysine were obtained similarly. Solutions used for NMR contained 1–4 mM protein in phosphate-buffered saline at pH 6.0 (uncorrected for isotope effects), 50 mM sodium azide, 4 mM [U-13H]thiotreitol, and 10% 2H2O in the H2O samples. Sample precipitation was observed at temperatures >40°C. H2O samples were prepared by lyophilizing three times and dissolving the residue in 99.996% 2H2O. The internal 1H chemical shift reference used was sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and indirect referencing was used for 15N (22).

NMR Spectroscopy. NMR experiments were run on a General Electric model OMEGA 500 spectrometer or Bruker model DMX-500. Quadrature detection was achieved by the States or States-time proportional phase incrementation.

Abbreviations: PH, pleckstrin homology; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; PFP2, phosphatidylinositol 4,5-bisphophate; RMSD, root-mean-square deviation.

†To whom reprint requests should be addressed.

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FIG. 1. Amino acid sequence and summary of observed and calculated NMR data related to secondary structure. The amino acid sequence of the protein is shown at the top, corresponding to residues 524–658 in the complete sequence of dynamin. Each subsequent row gives the value for the individual residues, with an appropriate scale range indicated by the left hand vertical bar. There are no observed values for positions 84–87. \( \Delta C^\alpha \), differences in chemical shifts between observed \(^{13}\text{C}^\alpha \) and expected values for amino acid type. Positive values are commonly correlated with an \( \alpha \)-helix, and negative values are correlated with \( \beta \)-strands (range, +5 to –5 ppm). \( \Delta C^\beta \), differences in chemical shifts between observed \(^{13}\text{C}^\beta \) and expected values for amino acid type. Negative values are commonly correlated with an \( \alpha \)-helix, and positive values are correlated with \( \beta \)-strands (range, +5 to –5 ppm). \( \log(t_{1/2} \text{HX}) \), \( \log(t_{1/2} \text{HX}) \), log of measured half-times of exchange for amide hydrogens with deuterium, with a maximum set to 100 min (range, 0–4). \( \log(t_{1/2} \text{HX}) \), product of \( \log(t_{1/2} \text{HX}) \) and \( \log(t_{1/2} \text{HX}) \) as a qualitative indicator of positions of secondary structural elements. Positive values are correlated with an \( \alpha \)-helix; negative values are correlated with \( \beta \)-strands (range, +30 to –30). \( d_{\text{HN}}(i, i+1) \), \( d_{\text{HN}}(i, i+1) \), \( d_{\text{HN}}(i, i+3) \), \( d_{\text{HN}}(i, i+3) \), \( d_{\text{HN}}(i, i+4) \), \( d_{\text{HN}}(i, i+4) \), \( 15N^1\text{H} \) NOEs, the ratio of the recorded intensities of spectra with and without heteronuclear NOEs. Missing values are from unassigned peaks or where overlap precludes precise integration. Positive values of 0.7–0.8 are appropriate to the expected \( r_\ell \) for a molecule of this size, while negative values indicate extensive flexibility (21). The expected theoretical value is 0.83 to –3.6; the range +1 to –1 is shown. RMSD, calculated RMSD for 16 calculated structures (bar graph) (range 0–8 Å). Line segments are the above \(^{15}\text{N}^1\text{H} \) NOEs multiplied by –1 and translated to a scale similar to the RMSDs. Heteronuclear NOEs are three-point smoothed from the previous row, and values unobserved are omitted. The concurrent rise and fall of the bars of RMSD and lines of heteronuclear NOE suggest that the high RMSD segments are more flexible than the rest of the molecule (see text). At the bottom, bars indicate the observed and calculated elements of secondary structure and their identities.

(TPPI) method. The water signal was suppressed by using selective on-resonance irradiation during a relaxation delay of 0.7–1.3 s and in NOE spectroscopy (NOESY) experiments also during the mixing period. Experiments were run at 25°C with sweep widths of 8000 and 1500 Hz for \(^1\text{H} \) and \(^{13}\text{N} \), respectively, unless indicated otherwise.

Homonuclear experiments were run in both \(^2\text{H}_2\text{O} \) and \(^2\text{H}_2\text{O} \) using standard pulse sequences and phase cycling. A range of \( t_1 \) increments from 200 to 512, each consisting of 2048 complex points was typically acquired with 32–128 scans per increment. In \(^2\text{H}_2\text{O} \) double-quantum filtered two-dimensional correlated spectroscopy spectra, 4096 points were acquired. Z-filtered homonuclear Hartmann–Hahn spectroscopy (HOHAHA) experiments used an MLEV-17 sequence for spin lock with mixing times of 8, 33, and 65 ms in \(^2\text{H}_2\text{O} \) and between 8 and 90 ms in \( \text{H}_2\text{O} \). NOESY spectra were recorded in \(^2\text{H}_2\text{O} \) with mixing times \( t_{\text{mix}} \) of 20, 50, 100, 150, and 200 ms and in \( \text{H}_2\text{O} \) with \( t_{\text{mix}} \) values of 50, 100, 150, and 200 ms. A three-dimensional NOESY heteronuclear multiple quantum coherence spectroscopy (NOESY-HMQC) and a small number of three resonance experiments were performed on a Bruker AMX-600 at Bruker Instruments, including CBCA(CO)NH, HN(CO)CA, and HNCA sets (23, 24).

Other heteronuclear experiments consisted of HMQC, HMQC-\( ^1\text{J} \), heteronuclear single quantum coherence spectroscopy, \(^{15}\text{N} \)-filtered HOHAHA and NOESY, two-and three-dimensional NOESY-HMQC, and three-dimensional HOHAHA-HMQC experiments. The \( t_{\text{mix}} \) values in NOESY-HMQCs were 100 ms, and in three-dimensional HOHAHA-HMQC, the spin lock was 30 ms. The three-dimensional spectra were recorded with a \( 32 \times 100 \times 1 \) k hypercomplex matrix, with 32 scans per increment. The amide hydrogen exchange rates were measured by following the intensity of crosspeaks in HMQC experiments after exchanging a full protonated, lyophilized sample with 99.996% \(^2\text{H}_2\text{O} \). Heteronuclear NOEs were measured using standard methods with water presaturation (21). This method is recognized to produce small errors from presaturation, but these are insignificant for the qualitative uses of this paper.

Signal processing typically consisted of a single zero-filling and polynomial baseline correction in \( \omega_1 \), NOESY crosspeak intensity was estimated from the volume and maximum height relative to the height of crosspeaks between hydrogens separated by a fixed distance, using XEASY and CALIBA packages (26).

Spin System and Sequential Assignment. Typing and linking of spin systems was accomplished by integrating fragments derived from the CBCA approach (23) with more conventional NOE-based assignment (27).

Structural calculations used DIANA with REDAC (28), with structurally significant constraints of 752 upper and 187 lower distance bounds (from \( \approx 1500 \) NOEs), 38 hydrogen bonds
chosen within strands of helix, with slowed exchange, and neighboring NOEs between strands, and 50 \( \phi, \psi \) constraints corresponding to conservative ranges of allowed angles, in those regions of strand or helix which were well defined (see Fig. 1). All peptide bonds were assumed to be trans. A final selection of 16 structures from 200 starting structures was done by using the lowest target functions (see Fig. 3 B and E). DIANA uses no assumptions about protein energetics, other than van der Waals repulsion; structures are unrefined and only adjacent coordinate rotation/translation for comparison purposes. Structures were aligned using XPLOR and displayed and analyzed with Biosym Technology's INSIGHTII package.

RESULTS AND DISCUSSION

The 125-amino-acid section of dynamin used is that shown in Fig. 1. This section was selected to obtain an entirely homogeneous preparation with minimal secondary proteolysis, as determined by mass spectrometry. A combination of two- and three-dimensional NMR experiments were used to identify and sequentially assign nearly all of the \( ^1\text{H}, ^{13}\text{C}, \) and \(^{15}\text{N} \) resonances in the backbone and \( C^\beta \) positions of the total 125-amino-acid residues, as shown in Fig. 1. Line broadening due to chemical exchange precluded the identification of NOEs or other connectivities from the amide resonances of unassigned residues, located in the putative loop region, residues 84–87.

Elements of secondary structure were defined by several NMR criteria as summarized in Fig. 1. Eight \( \beta \) strands were predicted by negative \( \Delta C^\alpha \) shifts, restricted exchange, and strong \( d_{\text{NN}}(i, i + 1) \) NOEs. Helical regions are identified by coincidence of strong \( d_{\text{NN}}(i, i + 1) \) NOEs, by NOEs to hydrogens three and four residues distant, and by CBCA shift values (29). One \( \alpha \)-helix is found for the sequence 103–115. A network of hydrogen bonds implied by eight slowly exchanging amides stabilizes the helix. In Fig. 2, the network of homonuclear NOEs defining the sheets is shown.

For calculated structures, there were no apparent sets of different topologies. The root-mean-square deviation (RMSD) between the set and its average is 0.94 \( \AA \) for the core segments (14–20, 32–37, 41–45, 55–57, 62–66, 75–81, 95–100, 103–115, 117–120). The precision of any structure derived without complete assignment of all hydrogens beyond the \( \beta \) position is necessarily limited; however, the general fold of the dynamin PH domain in solution is available from these structures.

In solution, dynamin PH consists of two major sheets, of strands 1–4 and 5–7. These sheets are composed of antiparallel strands, and run continuously into each other \( 1 \to 2 \to 3 \to 4 \) and \( 5 \to 6 \to 7 \). The sheets form a sandwich surrounding a hydrophobic core, with the strands of one sheet making an \( \approx 60^\circ \) crossing angle with those of the other sheet (Fig. 3A). The end of the second sheet (\( \beta 7 \) strand) leads via a tight turn (\( 7/1; \) Fig. 1) into a four-turn \( \alpha \)-helix. A further tight turn leads into a short \( \beta \)-strand, which crosses strand \( \beta 5 \). The negative heteronuclear \(^{15}\text{N}[^1\text{H}] \) steady-state NOE establishes that residues 1–10 and 123–125 are essentially without structure. The reduced positive NOEs in loops 1/2, 2/3, and 5/6 are readily interpreted as increased flexibility in these areas (Fig. 1), mimicked by the calculated RMSD for static structures (Figs. 1 and 3B). The unassigned residues in loop 6/7 preclude a similar interpretation of heteronuclear NOEs, but the technical difficulties in observing these residues may arise from conformational fluctuations in this region, leading to line broadening from exchange on an intermediate time scale. It seems likely that the overall changes in mobility reflected in increased RMSD and decreased heteronuclear NOEs in loops 1/2, 3/4, and 4/5 are effective also in loop 6/7, as suggested in many other cases (31).

![Fig. 2. Schematic diagram of the antiparallel \( \beta \)-sheet regions of the PH domain. Solid lines indicate observed long-range NOEs. Dashed bars represent NOEs that are expected but not unequivocally identified, due to resonance overlap or line broadening due to chemical exchange. Hydrogen bonds from slowly exchanging amide protons that are supported by at least one interstrand NOE are indicated by dashed lines. Strand 8 is a mini-\( \beta \)-strand, which crosses strand 5 at approximately right angles. It is illustrated here as a parallel sheet interaction. Italic single letters are amino acid residue designations.](image-url)

This topology of the fold in solution is identical to that observed in the crystal structure of dynamin PH (8) and similar to that proposed in solution for other PH domains. The dimerization in the crystal structure does not then appear to have major effects on the structure. RMSDs between the crystal structure and all solution structures are \( \beta 1, 0.74; \beta 2, 0.60; \beta 3, 0.64; \beta 4, 0.47; \beta 5, 0.79; \beta 6, 0.53; \beta 7, 0.60; \alpha 1, 0.44; \) and \( \beta 8, 1.28 \). The larger values for \( \beta 5 \) and \( \beta 8 \) RMSD between solution and crystal probably reflect minor changes caused by dimer formation, although these secondary structural elements are not directly at the interface. This study definitively identifies the length of the folded domain from the positive heteronuclear \(^{15}\text{N}[^1\text{H}] \) steady-state NOE determination for residues 10–121. The sequence of the expressed spectrin PH was significantly shorter in the C terminus (7). The sequence of the expressed pleckstrin-N PH contains a "histidine tag" immediately after the equivalent of position 123 in dynamin PH (3). Such an unnatural insertion may alter the local conformation. In these other solution structures, the C-terminal contacts of the helix are not reported. In contrast, the structures of dynamin PH contain a mini-\( \beta \)-strand (\( \beta 8 \)), which definitely establishes the position of the helix close to the rest of the molecule. Assuming this to be a general feature of PH domains, the long C-terminal helix then appears to have a totally distinct structure from that of proteins like retinol binding protein; the latter class normally has a helix–turn–helix at the C terminus (25). Nonetheless, the suggestion that PH domains may be involved in binding to lipids (2, 3) is intriguing, and dynamin PH has a binding affinity to PIP\(_2\) (J. Zheng and D.C., unpublished results), similar to that of pleckstrin-N PH (3). However, so far there is no evidence that lipids including PIP\(_2\) bind to the dynamin PH domain or to other PH domains specifically. In
Fig. 3. Ribbon (A–C and E) and solid-surface (D) representations of dynamin PH domain tertiary structure. Only residues 11–123 are shown. (A) View facing the junction of sheets of one calculated structure. The color coding is as follows: the α-helix is blue, the strands β1–β8 are 1, red; 2, pink; 3, salmon; 4, gold; 5, green; 6, magenta; 7, light blue; 8, cyan; the tight turns are white, the loops and the termini are grey. (A–D) Same single structure, closest to the average structure, as generally suggested (30). (B) Ribbon with the width proportional to local backbone RMSD to illustrate the precision of structure determination. The molecule orientation is similar to A. The similarity of RMSD and heteronuclear $^{15}$N($^1$H) NOE distribution suggests that the high RMSD/low NOE sections are dynamic. The smallest ribbon width is limited to 0.2 Å. (C) View facing loops 1/2, 3/4, 5/6, and 6/7 of dynamin PH domain. The color code is the same as that in A. (D) Charge distribution on the surface of dynamin PH domain. Positive areas are colored in blue, negative in red, and neutral in white to grey. Positive charges are labeled. With the exception of K53, all of them are extensively conserved in PH domains. The molecule orientation is similar to that in C. (E) Superimposition of 16 calculated structures demonstrates disorder/flexibility in the loop regions 1/2, 3/4, and 6/7 and in the termini. The color coding is the same as in A and C.
pleckstrin- N PH, the area of a possible PIP2 binding site has been suggested to be in the 1/2 and 2/3 loops, especially the lysine and arginine side chains. Dynamin PH presents a similarly charged surface (Fig. 3E), which can probably accommodate multiple phosphorylated ligands. In this respect, the demonstration of specificity and physiological significance is needed to discriminate the possible role of PH in PIP2 binding from the suggested, hypothetical role of PH in recognizing, for example, phosphorylated serine and threonine sites (4). The wide sequence variability and range of association of PH domains with other functional domains argues for a broader role than recognizing a ubiquitous lipid. The determination of larger number of PH domain structures may assist in identifying common areas of possible ligand sites.

Note Added in Proof. Since submission of this article, two publications have appeared describing a 120- amino-acid residue segment corresponding to residues 2-121 of the sequence used in this paper. One paper (32) describes the 2.8 Å crystal structure, and the other (33) describes the solution structure from NMR measurements. These structures, that of ref. 8, and that of this paper are in general agreement.

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