Pyrin domain (PYD)-containing proteins are key components of pathways that regulate inflammation, apoptosis, and cytokine processing. Their importance is further evidenced by the consequences of mutations in these proteins that give rise to autoimmune and hyperinflammatory syndromes. PYDs, like other members of the death domain (DD) superfamily, are postulated to mediate homotypic interactions that assemble and regulate the activity of signaling complexes. However, PYDs are presently the least well characterized of all four DD subfamilies. Here we report the three-dimensional structure and dynamic properties of ASC2, a PYD-only protein that functions as a modulator of multidomain PYD-containing proteins involved in NF-κB and caspase-1 activation. ASC2 adopts a six-helix bundle structure with a prominent loop, comprising 13 amino acid residues, between helices two and three. This loop represents a divergent feature of PYDs from other domains with the DD fold. Detailed analysis of backbone 15N NMR relaxation data using both the Lipari-Szabo model-free and reduced spectral density function formalisms revealed no evidence of contiguous stretches of polypeptide chain with dramatically increased internal motion, except at the extreme N and C termini. Some mobility in the fast, picosecond to nanosecond timescale, was seen in helix 3 and the preceding α2–α3 loop, in stark contrast to the complete disorder seen in the corresponding region of the NALP1 PYD. Our results suggest that extensive conformational flexibility in helix 3 and the α2–α3 loop is not a general feature of pyrin domains. Further, a transition from complete disorder to order of the α2–α3 loop upon binding, as suggested for NALP1, is unlikely to be a common attribute of pyrin domain interactions.

Inflammatory caspases such as caspase-1 play an essential role in innate immune responses to infection by regulating the processing of pro-inflammatory cytokines interleukin-1β and interleukin-18 into their mature, secreted forms (1, 2). Tight regulation of the production of these cytokines is required to maintain the homeostasis of host tissues. Excessive interleukin-1β production and chronic inflammation are hallmarks of many autoimmune diseases that present both systemically and within the central nervous system, including rheumatoid arthritis and multiple sclerosis (3, 4).

Similar to initiator caspases involved in apoptosis, the activation of inflammatory caspases requires their recruitment into a multiprotein signaling complex, which promotes dimerization and cleavage to produce the active enzyme via an induced proximity mechanism (2, 5). Recently, models for caspase-1 activation have been proposed whereby inflammatory stimuli promote the formation of molecular platforms referred to as inflammasomes (6). The NALP1 inflammasome induces the activation of both caspase-1 and caspase-5 through the formation of a complex that also contains the proteins NALP1 and ASC (7). Similarly, the NALP2/NALP3 inflammasome is involved in the activation of caspase-1 through the recruitment of NALP2 or NALP3, ASC, Cardi-nal, and caspase-1 (8).

The components of the inflammasome encode multiple protein-protein interaction domains, including the pyrin domain (PYD),2 caspase recruitment domain (CARD), and a nucleotide binding and oligomerization domain, that promote homotypic interactions between molecules to facilitate assembly. For instance, the adaptor protein ASC has a dual-domain structure consisting of an N-terminal PYD and a C-terminal CARD that enables it to function as a bridge between the PYD of various NALPs and the CARD of caspase-1 (2, 7). Inflammasome assembly is modulated by other PYD family members, including pyrin and ASC2, which interact with the inflammasome via their PYD domains to promote or inhibit activity (9–11). Furthermore, hereditary mutations in key components of the inflamma-some are thought to contribute to several types of autoinflammatory disease. For example, an increased incidence of familial Mediterranean fever has been associated with muta-

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.

‡ The atomic coordinates (code 2HM2) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: PYD, pyrin domain; CARD, caspase recruitment domain; DD, death effector domain; DD, death domain; NOE, nuclear Overhauser effect; NOEY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; RDC, residual dipolar coupling; r.m.s.d., root mean square deviation.
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tions in pyrin, whereas mutations in NALP3 have been linked to familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and neonatal-onset multiple-system inflammatory disease (12, 13).

The molecular mechanisms by which PYD-containing molecules regulate inflammatory responses are under intense investigation. Recent studies have identified a small protein, ASC2 (also known as POP1 and ASC1), as a regulator of multidomain PYD-containing proteins involved in NF-kB and caspase-1 activation (10, 14). ASC2 consists solely of a PYD and appears to function as a dominant-negative inhibitor, similar to the CARD-only proteins ICEBERG and pseudo-ICE, which suppress caspase-1 activation (15–17), or the death effector domain (DED)-only FLIP proteins in regulating apoptosis by affecting the recruitment of caspase-8 to tumor necrosis factor family death receptors (18). Consistent with this notion, ASC2 associates with the adapter protein ASC to inhibit its ability to collaborate with pyrin and NALP3 in NF-kB and caspase-1 activation (10).

Interestingly, ASC2 closely resembles the pyrin domain of ASC (64% sequence identity), and the genes encoding these proteins are located on nearby regions of chromosome 16, suggesting that they arose by gene duplication (10). The predominant expression of ASC2 in monocytes, macrophages, and granulocytes (10) further supports a role for ASC2 in the regulation of inflammatory responses.

Despite the importance of PYD-containing proteins in the regulation of inflammation and apoptosis, the underlying mechanisms remain largely unknown. At present, there is only limited structural information on the isolated PYDs of ASC and NALP1 (19, 20). The structure of ASC PYD was found to conform to the canonical fold of the death domain (DD) superfamily comprising six antiparallel \( \alpha \)-helices, which is also shared by the DD, DED, and CARD subfamilies (21). In contrast, the region that normally corresponds to the third helix in the DD fold was found to be completely disordered in NALP1 PYD (20). This has fueled speculation that PYD interactions may involve a conformational change upon binding, and that the folding/unfolding transition of helix 3 may be an important determinant of the function and disease-related dysfunction of this domain (20, 22, 23).

In an effort to further characterize the molecular basis of PYD interactions and the contribution of dynamics, we have determined the three-dimensional solution structure and dynamic properties of ASC2 using NMR spectroscopy. ASC2 adopts a core six-helix bundle structure characteristic of the DD superfamily. As in other members of the PYD subfamily, including ASC and NALP1, ASC2 is characterized by the presence of an insertion between helices 2 and 3 that forms a prominent, exposed loop of non-regular secondary structure. This feature is divergent from other members of the DD superfamily. \( ^{15} \)N relaxation data indicate that ASC2 is a globular protein with an approximately isotropic diffusion tensor and a rotational correlation time of 6.2 ns. A detailed analysis of backbone \( ^{15} \)N relaxation data using the Lipari-Szabo model-free formalism (24–26) as well as the reduced spectral density function approach (27) revealed that the \( \alpha 2-\alpha 3 \) loop and the third \( \alpha \)-helix (\( \alpha 3 \)) displayed a marginally greater degree of conformational disorder than the other five \( \alpha \)-helices. This was in stark contrast to the complete disorder observed in the \( \alpha 3 \) region of NALP1 PYD (20). This observation indicates that the mechanism of PYD interactions, namely a local disorder/order transition upon binding as proposed for NALP1 PYD (20, 22), may be a unique mode of interaction in that case and not a general feature of interactions involving PYDs.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Full-length ASC2 (residues 1–89) was subcloned into the bacterial expression vector pQE-30 (Qiagen), which produces the recombinant protein with an N-terminal His\(_6\) tag. Uniformly \( ^{15} \)N- and \( ^{15} \)N/\( ^{13} \)C-labeled proteins were overexpressed in *Escherichia coli* BL21 cells in minimal media containing \( ^{15} \)NH\(_4\)Cl (1 g/liter) with or without \( ^{13} \)C-glucose (2 g/liter) as the sole nitrogen and carbon sources, respectively. Cells were grown at 37 °C to an optical density (A\(_{600,\text{nm}}\)) of \( \sim 1.0 \) and then induced with 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside for 5 h. After harvesting, the cells were suspended in 50 mM Tris buffer (pH 8.0), 1 M NaCl, 30 mM imidazole, and 10 mM benzamidine hydrochloride, lysed by sonication, and centrifuged. The protein was purified using Ni\(^{2+}\) affinity chromatography and judged to be \( \geq 95% \) pure by SDS-PAGE analysis. Samples for NMR contained 1 mM protein in 10 mM sodium phosphate buffer (pH 7.3) and 140 mM NaCl in D\(_2\)O. NMR spectroscopy—All NMR experiments were acquired at 25 °C on a Bruker Avance 600-MHz spectrometer equipped with a z-shielded gradient triple resonance probe. The backbone- and side-chain \( ^{1} \)H, \( ^{13} \)C, and \( ^{15} \)N resonances of the protein were assigned using CBCA(CO)NH, HNCA, HN(CA)CO, HBHA(CO)NH, (C(CO)NH, H(CCO)NH and three-dimensional HCCH-TOCSY experiments (28). NOE-derived distance restraints were obtained from \( ^{15} \)N- or \( ^{13} \)C-separated three-dimensional NOESY spectra (mixing times of 110 and 120 ms, respectively). \( ^{1} \)H-NH, \( ^{1} \)N-H\(_{\text{NH}}\), and \( ^{1} \)N-H\(_{\text{CA}}\) coupling constants were measured by quantitative J correlation spectroscopy (29). \( ^{1} \)D-NH residual dipolar couplings were measured on a \( ^{15} \)N-labeled sample partially aligned in 5% polyethylene glycol (C12E5)/hexanol with a surfactant to alcohol ratio of 0.96 (30). \( ^{15} \)N-H\(^{14} \)N splittings were measured on the isotropic and partially aligned samples using two-dimensional \( ^{1} \)H-\( ^{15} \)N HSQC-IPAP experiments (31). Slowly exchanging amide protons were identified from a series of two-dimensional \( ^{1} \)H-\( ^{15} \)N HSQC spectra recorded after the buffer was changed to D\(_2\)O. NMR spectra were processed with NMRPipe/NMRDraw (32) and analyzed using PIPP and STAPP (33).

**Structure Calculations**—NOEIs within the protein were grouped into four distance ranges, 1.8–2.7, 1.8–3.3, 1.8–5.0, and 1.8–6.0 Å, corresponding to strong, medium, weak, and very weak intensities. Distances involving methyl groups, aromatic ring protons, and non-stereospecifically assigned methylene protons were represented as a \( \sum f^{-6} \) sum (34). \( 80 \) \( ^{1} \)H and \( 48 \) \( ^{1} \)C angle restraints were derived from an analysis of \( ^{1} \)N\(_{\text{HCO}}\) and \( ^{1} \)N\(_{\text{HCO}}\) coupling constants (29), and 69 \( \psi \) angle restraints were determined by chemical shift data base analysis using the program TALOS (35). The minimum range employed for \( \phi \), \( \psi \), and \( \chi_1 \) torsion angle restraints
was ± 30°. Hydrogen bond distance restraints (\(r_{\text{NH-O}} = 1.5–2.8\ \text{Å}\) and \(r_{\text{N-O}} = 2.4–3.5\ \text{Å}\)) were added during the final stages of refinement for residues within α-helices as derived from an analysis of amide proton exchange, \(^{13}\)Cα chemical shifts, and characteristic NOE patterns. The structures were calculated with the program XPLOR-NIH 2.11.2 (36) using a simulated annealing protocol incorporating pseudo-potentials for \(^{3}\)JH,H coupling constants (37), secondary \(^{13}\)Cα and \(^{13}\)CB chemical shifts (38), and residual dipolar couplings (39). The initial axial and rhombic components of the alignment tensor were estimated from the normalized distributions of \(^{13}\)C chemical shifts (38), and residual dipolar couplings (39).

Following the grid search strategy (41), optimum values of 0, 0.55 were calculated and used for subsequent structure generation. There were no hydrogen-bonding, electrostatic, or 6–12 Lennard-Jones empirical potential energy terms in the target function. The final ensemble of 20 NMR structures was selected on the basis of lowest energy and least number of restraint violations; these structures had no distance restraint violations >0.5 Å and no dihedral angle violations >5°. Structure quality was assessed with PROCHECK-NMR (42), and structures were displayed and analyzed using MolMol (43) and GRASP (44).

**Backbone Relaxation Experiments**—Relaxation experiments were performed at 25 °C using either a Varian Inova 600 MHz spectrometer or a Bruker Avance 800 MHz spectrometer with cryoprobe. Both spectrometers were equipped with triple-resonance probes capable of applying pulsed field gradients along the z-axis. A complete set of \(R_1, R_2,\) and \(^{1}H^{15}N\) NOE measurements (45) were collected at 600 and 800 MHz. Recycle delays of 1.5 s were used in all experiments. The following relaxation delays were used to measure the \(R_1\) values at 600 MHz: 7 (×2), 102, 202, 302, 502, 702 (×2), and 1002 ms; and at 800 MHz: 10 (×2), 20, 50, 100, 200, 300, 400, 500 (×2), 700, 750, and 1000 (×2) ms. For the \(R_2\) measurements, the following variable relaxation delays were used at 600 MHz: 10 (×2), 30, 50, 70, 110, 130, 170 (×2), and 210 ms; and at 800 MHz: 4 (×2), 32, 52, 72 (×2), 102, 152, 172, and 220 (×2) ms. \(R_{\text{pp}}\) values were obtained at 600 MHz using identical relaxation delays as in the \(R_2\) experiment above. A spin-lock field (\(\Delta\omega_{\text{SL}}\)) of 1500 Hz was used to obtain sample heating effects similar to those in the \(R_2\) measurements above to allow an accurate comparison between the two rates. The offset-corrected \(R_{\text{pp}}\) values were obtained from the measured \(R_{\text{pp}}\) using the following equation,

\[
R_{\text{pp}} = \frac{R_{\text{pp}} - R_{1}\cos^2 \beta}{\sin^2 \beta} \quad (\text{Eq. 1})
\]

with \(\beta = \tan^{-1}(\omega_{\text{SL}}/\Delta\omega)\), and \(\Delta\omega\) is the offset from the carrier frequency.

All data were processed using the NMRPipe software suite and visualized with NMRDraw (32). Peak intensities were quantitated using the nlinLS module of the NMRPipe suite, and the relaxation rates \(R_1, R_{\text{pp}},\) and \(R_2\) were determined by fitting the peak intensity \(I(t)\) to a single exponential function given by \(I(t) = I_0 e^{-rt}\) (\(R = R_1, R_{\text{pp}},\) and \(R_2\)) using in-house programs that utilized the ODRPACK (46) libraries. Errors reported correspond to the 68.3% bounds determined from the inverse of the covariance matrix of the fits and include both the random and model selection errors.

Steady-state \(^{1}H^{15}N\) NOE values were obtained by recording two spectra with and without a 3.5-s period of proton saturation. The error (\(\sigma_{\text{NOE}}\)) was determined using the following equation,

\[
\sigma_{\text{NOE}} = \frac{I_{\text{sat}}}{I_{\text{unsat}}} \left(\frac{\sigma_{\text{sat}}}{I_{\text{sat}}} + \frac{\sigma_{\text{unsat}}}{I_{\text{unsat}}}\right)^{1/2} \quad (\text{Eq. 2})
\]

where \(I_{\text{sat}}\) and \(I_{\text{unsat}}\) represent the measured intensities of a particular resonance in the presence and absence of proton saturation, and \(\sigma_{\text{sat}}\) and \(\sigma_{\text{unsat}}\) represent the root mean square variation in the noise in empty spectral regions of the spectra with and without proton saturation.

**Analysis of the Hydrodynamic Properties**—The hydrodynamic properties of ASC2 were determined using the program DYNAMICS version 2.0, which utilized methods described previously (47). Residues with \(^{1}H^{15}N\) NOE values smaller than 0.6 at 600 MHz and 0.7 at 800 MHz, and residues with significant exchange contributions as determined by their \(I_{\text{prod}}\) values, and \(R_2/R_1\) ratios, described previously (48), were excluded in the procedure to determine the overall diffusion tensor. Errors (68.3% confidence limits) in the principal values and orientation of the rotational diffusion tensor were obtained from the analytically determined inverse covariance matrix of the fits. Model selection between the fully anisotropic, axially symmetric, and isotropic models was performed using the statistical F-test. Probabilities (P%), indicating the possibility that the improvement in fits on increasing model complexity was obtained by chance, were calculated for each of the pairs of models, fully anisotropic/axially symmetric and axially symmetric/isotropic. Values of \(P > 5\%\) resulted in the less complex model being selected.

**Lipari-Szabo Model-free Analysis**—An analysis of the microdynamic motional parameters using the Lipari-Szabo formalism (24) was performed utilizing the program DYNAMICS (26) using the \(R_1, R_2,\) and \(^{1}H^{15}N\) NOE data at 600 and 800 MHz. Several runs were carried out using various subsets of the relaxation rates to determine the stability of the analysis and the robustness of the selected models and estimated microdynamic parameters with respect to completeness of datasets. Errors (68.3% confidence limits) in the microdynamic parameters were obtained from the analytical inverse covariance matrices of the fits to include both the random error and the model selection error, the latter being dominant in multidimensional fits as in the present case. The Lipari-Szabo microdynamic parameters (including the overall correlation time, \(\tau_c\), obtained from the hydrodynamic analysis described above) were optimized by minimizing the \(\chi^2\) value determined by Equation 3,

\[
\chi^2 = \sum_r \sum_x \sum_j \left(\frac{X_{\text{obs}}^{ij} - X_{\text{calc}}^{ij}}{\sigma_{\text{obs}}^{ij}}\right)^2 \quad (\text{Eq. 3})
\]

The summation runs over the two fields \(f\) (600 and 800 MHz), the measured relaxation rates \(X^{ij}\) \((R_1, R_2,\) and \(^{1}H^{15}N\) NOE or...
a subset thereof), and over the residues $j$ (2–90). Models used and computational strategies employed have been discussed at length previously (26).

Reduced Spectral Density Functions—The reduced spectral density functions $J_{\omega}$, at a given field strength ($f = 600$ or 800 MHz) are given by,
\[ J'(\omega_n)_f = \frac{R_{1,f}}{3[c_f^2 + d^2]} \]  
(Eq. 4)

\[ J'(0.87\omega_n)_f = \frac{HF_f}{d^2} \]  
(Eq. 5)

\[ R_{1,f} = R_{1} - 7 \left( \frac{0.87}{0.921} \right)^2 HF_f \]  
(Eq. 6)

\[ R_{2,f} = R_{2} - \frac{13}{2} \left( \frac{0.87}{0.955} \right)^2 HF_f \]  
(Eq. 7)

\[ HF_f = -\gamma_n \frac{\gamma_B \Delta \sigma}{5} \frac{1}{\gamma_H} R_{1,f} \]  
(Eq. 8)

and where

\[ c_f = \frac{\gamma_n \gamma_B \Delta \sigma}{3}, \quad d = -\frac{\mu_0}{4\pi} \frac{\gamma_n \gamma_B \Delta \sigma}{2r_{ni}^3}. \]

All other symbols have their usual meaning. Values of \( \gamma_{NS} = 1.02 \) Å and \( \Delta \sigma = -160 \) ppm were used as in the Lipari-Szabo analyses discussed above. The spectral density function at zero-frequency was obtained from the following equation (27),

\[ J(0) = 2 \pi [R_{2,600} - \kappa R_{2,600}] - \frac{3d^2}{2} \left( J'(\omega_n)_{600} - \kappa J'(\omega_n)_{600} \right) \]

\[ - \frac{3c_{600}^2}{2} \left( J'(\omega_n)_{600} - \kappa J'(\omega_n)_{600} \right) \]

\[ - \frac{13d^2}{2} \left( \frac{0.87}{0.955} \right)^2 \left( J'(\omega_n)_{600} - \kappa J'(\omega_n)_{600} \right) \]  
(Eq. 9)

and the exchange contribution to \( R_2 \) at 600 MHz (\( R_{ex} \) at 600 MHz) is given by Equation 10,

\[ R_{ex,600} = R_{2,600} - \frac{1}{\pi} \left[ c(600)^2 + d^2 \right] J(0) - \frac{3}{4\pi} c_{600}^2 + d^2 \left( J'(\omega_n)_{600} \right) \]

\[ - \frac{13d^2}{4\pi} \left( \frac{0.87}{0.955} \right)^2 \left( J'(\omega_n)_{600} \right) \]  
(Eq. 10)

and \( \kappa = (B_{0,600}/B_{0,0})^2 \). The \( R_{ex} \) contribution scales as the square of the static magnetic field \( B_0 \).

Coordinates—The coordinates of the 20 ASC2 NMR structures have been deposited in the RCSB protein data bank with accession number 2HM2.

RESULTS

Three-dimensional Structure of ASC2—Members of the death domain superfamily often exhibit poor solubility due to self-association that makes detailed NMR-based structural analysis difficult (15, 49, 50). To date, structural studies of pyrin domains have also been hampered by these characteristics. Attempts to solve the NALP1 PYD structure were precluded by poor solubility of the isolated domain (51) but subsequently overcome by using an N-terminal protein G (B1 domain) fusion as a solubility-enhancing tag (20). The structure of the ASC PYD had to be determined at pH 3.7 from refolded protein (19), because the protein precipitated at neutral pH. In contrast, ASC2 was readily overexpressed in E. coli in a soluble form, was monomeric in solution as judged by size-exclusion chromatography, and gave high quality NMR spectra at neutral pH (Fig. 1A). Thus, ASC2 represented an ideal candidate for detailed studies of pyrin domain structure and backbone dynamics.

The backbone and side-chain resonances of ASC2 were assigned using standard heteronuclear multidimensional NMR experiments (28). Several discrepancies with the previously published resonance assignments (52) were observed and resolved by careful analysis of through-bond connectivities in the CBCA(CO)NH, HNCACB, HNCO, and HN(CA)CO spectra, as well as sequential NOE connectivities in the \(^{13}N\) NOESY-HSQC. Differences in sequential assignment of ASC2 were determined for residues Thr-3, Lys-4, Pro-17, Glu-18, Thr-29, Val-30, Pro-40, and Arg-41 (Fig. 1A), and analysis of the Ca secondary shifts of ASC2 indicated the presence of six helices (Fig. 1B). Interestingly, several residues flanking helix 3 showed significant line-broadening including Gly-42, Gly-45, and Gln-46, suggesting that conformational exchange is present in this region of the molecule. Notably, in NALP1 cis-trans isomerization of the Thr-41 to Pro-42 peptide bond was observed (20). However, in ASC2 the presence of a strong sequential H\(^{\alpha}_{39}\)H\(^{\delta}_{40}\) NOE and absence of an H\(^{\alpha}_{39}\)H\(^{\delta}_{40}\) NOE indicates that the Ile-39 to Pro-40 peptide bond was in the trans conformation. This does not preclude the presence of a small, undetectable population of the cis conformer in slow exchange with the trans (major and detectable) conformer resulting in line-broadening effects in this region.

The three-dimensional structure of ASC2 was determined from a total of 1778 NMR-derived restraints, including 1338

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>NMR structural statistics of ASC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without RDCs</td>
<td>With RDCs</td>
</tr>
<tr>
<td>r.m.s.d. values from experimental restraints</td>
<td></td>
</tr>
<tr>
<td>Distance restraints (Å) (1338)</td>
<td>0.041 ± 0.002</td>
</tr>
<tr>
<td>1(^{C}C) chemical shifts (ppm)</td>
<td>1.21 ± 0.02</td>
</tr>
<tr>
<td>1(^{C}H) (87)</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>3(^{C}H) (83)</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Dipolar angle restraints (°) (197)</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>1(^{J}HN) residual dipolar couplings (Hz) (73)</td>
<td>0.78 ± 0.11</td>
</tr>
<tr>
<td>Deviations from idealized covalent geometry</td>
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</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.0036 ± 0.0001</td>
</tr>
<tr>
<td>Angles (°)</td>
<td>0.55 ± 0.01</td>
</tr>
<tr>
<td>Improper (°)</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Coordinate precision (Å)</td>
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</tr>
<tr>
<td>Backbone (residues 3–87)</td>
<td>0.44 ± 0.09</td>
</tr>
<tr>
<td>All non-hydrogen atoms (residues 3–87)</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Ramachandran plot statistics</td>
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<tr>
<td>Most favored (%)</td>
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<tr>
<td>Additionally allowed (%)</td>
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<tr>
<td>Generously allowed (%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Disallowed (%)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* The interproton distance restraints comprised 286 intraresidue, 339 sequential (\( i-j=1 \)), 415 medium range (\( 1<i-j<5 \)), 236 long range (\( |i-j|>5 \)), and 62 hydrogen bond restraints.

* The torsion angle restraints comprised 80 \( \phi \), 69 \( \varphi \), and 48 \( \chi_1 \).

* The precision of the coordinates is defined as the average atomic r.m.s. difference between the 20 individual simulated annealing conformers and the mean coordinates for residues 3–87.

* PROCHECK_NMR (Laskowski et al. (42)) was used to assess the overall quality of the structures.
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NOEs, 197 dihedral angle restraints, 73 $^3J_{\text{NH}}$ coupling constants, and 170 $^1\text{H}^1\text{C}/^1\text{H}^1\text{C}\beta$ secondary shift restraints. All structures exhibit good geometry, with no violations of distance restraints >0.5 Å and no dihedral angle violations >5° (Table 1). The ASC2 structure is well defined by the NMR data with atomic r.m.s.d. values about the mean coordinate positions of the backbone atoms (N, Ca, and C') and all heavy atoms for residues 3–87 of 0.44 ± 0.09 Å and 1.00 ± 0.07 Å, respectively.

ASC2 adopts a six-helical bundle fold, characteristic of the core structure of the DD superfamily (Fig. 2, A and B). The residues forming the six α-helices are 3–12, 17–27, 41–44, 49–60, 62–75, and 79–86. These helices are connected by short loops with the striking exception of along segment of residues 3–87. Residue numbers for every tenth residue are labeled. B, ribbon diagram of the lowest energy structure of ASC2 illustrating the six α-helices (cyan) comprising residues 3–12, 17–27, 41–44, 49–60, 62–75, and 79–86. The α2–α3 loop is colored in magenta. C, distribution of the r.m.s.d. in backbone coordinates between the ASC2 conformers and the average structure.

FIGURE 2. Three-dimensional structure of ASC2. A, ensemble of 20 NMR-derived structures of ASC2 superimposed over the backbone atoms (N, Ca, and C') of residues 3–87. Residue numbers for every tenth residue are labeled. B, ribbon diagram of the lowest energy structure of ASC2 illustrating the six α-helices (cyan) comprising residues 3–12, 17–27, 41–44, 49–60, 62–75, and 79–86. The α2–α3 loop is colored in magenta. C, distribution of the r.m.s.d. in backbone coordinates between the ASC2 conformers and the average structure.

from higher local r.m.s.d. values (Fig. 2C) and a rapid solvent exchange of the amide protons in these regions of the protein. The increased rates of amide proton exchange for residues within helices 3 and 6 correlates with a higher degree of solvent accessibility for these secondary structural elements.

Structure Validation Using Residual Dipolar Couplings—Although the overall folds of DD superfamily members are similar, there is often considerable variation in the location and relative orientation of the six helices, even within the same family (21). Consequently, there remains some uncertainty regarding the accuracy of NMR structures determined using only short range NOE-derived distance and dihedral angle restraints with respect to helical packing and orientation. For these reasons, it is valuable to provide a refined structure of ASC2 with accurate and unambiguous helical orientations.

The structure of ASC2 was further refined using additional restraints derived from RDCs. 83 $^3D_{\text{NH}}$ RDCs were measured on a $^1\text{H}_2^1\text{N}$-labeled sample partially aligned in polyethylene glycol/hexanol media. The initial axial ($D_a = -6.19$ Hz) and rhombic ($R = 0.59$) components of the alignment tensor were estimated from the normalized distribution of these RDCs (40). Following the grid search strategy (41), optimum values of $D_a (-6.5$ Hz) and $R (0.55)$ were calculated and used for subsequent structure generation. Fig. 3 shows the representative ASC2 structure ensembles generated with or without RDC data, and the structural statistics are summarized in Table 1. The inclusion of RDC restraints did not significantly alter the overall topology or the precision of the structural ensemble (Fig. 3, A and B), indicating that the structure of ASC2 was already well defined by the short range NOE-derived distance restraints and dihedral angle restraints. Moreover, the agreement with the other experimental data and the covalent geometry was only marginally affected by the incorporation of the RDCs (Table 1). The RDC values predicted using the coordinates generated without RDC restraints also fit quite well to the experimental RDC data (Fig. 3C), with a correlation coefficient of 0.836 between the calculated and observed RDC data. However, as expected the RDC-refined structures agree significantly better with the observed RDC values, with a correlation coefficient of 0.993 (Fig. 3D).

As observed for the structures calculated without RDCs, several regions of ASC2 remain less well defined in the RDC-refined structures, including the α2–α3 loop and helices 3 and 6 (Fig. 3A). For the most part, the helical orientations of ASC2
obtained with or without RDC data are also indistinguishable from each other. The main exception is a slight alteration in the relative orientation of helix 6 so that it is more closely associated with helix 1 and the core of the protein in the RDC-refined structures (Fig. 3, A and B).

Comparison with ASC and NALP1—The overall fold of ASC2 resembles the structures of the ASC and NALP1 PYDs (19, 20), the only other pyrin domain structures that have been determined to date. In particular, the ASC2 and ASC PYD structures are very similar, with an r.m.s.d. of 1.5 Å for Ca atoms of residues 1–88 (Fig. 4A). The NALP1 PYD contains only five α-helices and represents a significant departure from the canonical fold of the DD superfamily. Consequently, ASC2 is superimposed with the PYD of NALP1 with an r.m.s.d. of 2.8 Å over 84 aligned Ca atoms. In comparison with ASC and ASC2, the third helix in NALP1 is replaced by a flexibly disordered loop. The orientation of the α2–α4 loop in NALP1 was poorly defined by the NMR data, and 1H15N NOEs suggested that this disorder was due to inherent flexibility in this region of the protein (20). The NALP1 PYD also exhibits differences in the length of helices 1 and 6, with both being significantly shorter than in the other PYD structures.

The unexpected observation that helix 3 is unfolded in NALP1 has fueled speculation that pyrin domain interactions may involve a conformational change upon binding (20, 22, 23). Given the importance of helix 3 in mediating protein-protein interactions within the DD superfamily (21, 53, 54) and the possibility that flexibility in this region may play a role in the interaction and function of PYDs, we have also examined the dynamic properties of ASC2 using NMR spectroscopy.

Of the 88 residues in ASC2 (excluding M1), three were prolines (17, 31, and 40), residues Gln-34 and Tyr-64 overlapped, and the relaxation data for these residues could not be analyzed accurately. In addition, Gly-42, Gly-45, and Gln-46 were broad and excluded from the analysis. The relaxation data for Arg-41 and Gln-80 were also excluded from analysis due to large errors in peak fitting. Thus the analysis of R1, R2, and heteronuclear 1H15N NOEs at 600 and 800 MHz for a total of 78 residues (Fig. 5 and Table 2) revealed a protein with an axially symmetric diffusion tensor with a Dpar/Dperp value of 1.10 ± 0.03 from an analysis of relaxation data at 600 MHz (1.15 ± 0.07 at 800 MHz, supplemental Table S1). However, for a diffusion anisotropy this small, use of an isotropic model in the analysis of relaxation rates in terms of microdynamic parameters using the Lipari-Szabo model-free formalism would not be expected to lead to significant errors. Thus, an isotropic diffusion tensor was uti-
NMR Structure and Dynamics of ASC2

FIGURE 4. Comparison of the ASC2, ASC, and NALP1 pyrin domains. A, cylinder drawing showing the superposition of ASC2 (red) with the pyrin domains of ASC (yellow) (19; PDB accession code 1UCP) and NALP1 (blue) (20; PDB accession code 1PN5). The third helix in NALP1 PYD is replaced by a flexibly disordered loop. In both cases the residues used to superimpose the structures were identified using the three-dimensional structure comparison program DALI (65). B, structure-based sequence alignment of the pyrin domains from ASC2, ASC, and NALP1. Boxes indicate the positions of helices in ASC2, and the helices for ASC and NALP1 are underlined. Residues buried within the hydrophobic core of ASC2 (<10% solvent accessibility) are shown in gray, and hydrophobic residues in the α2–α3 loop are shown in blue.

lized in the analysis. The optimized rotational correlation time (after the Lipari-Szabo analysis) was found to be 6.2 ns.

Analysis of the microdynamic parameters revealed that the generalized order parameter (S^2) values were uniformly high except at the extreme N and C termini (Fig. 6A), indicating an extremely well ordered structure on the picosecond to nanosecond timescale. The average S^2 value (0.93 ± 0.03) for the helices (excluding α3) was only marginally higher than for the residues in the α2–α3 loop and α3 (residues 28–45, 0.90 ± 0.05), indicating that this region was no more mobile on the picosecond to nanosecond timescale than the other helical regions. The largest R_{ex} values occurred in the α5–α6 loop (Arg-77: 3.2 ± 0.3 s⁻¹) and at the C terminus (Arg-87: 3.0 ± 0.4 s⁻¹). Some smaller R_{ex} were also seen in the α4–α5 loop (Tyr-61: 1.9 ± 0.4 s⁻¹, Asp-63: 2.1 ± 0.2 s⁻¹). No R_{ex} values were seen in the α2–α3 loop or in α3 itself (however, residue Gly-42 was very broad indicating the presence of conformational exchange contributions to the spin-spin relaxation rates). Thus from an analysis of the relaxation rates using the Lipari-Szabo formalism, no evidence was found of extensive disorder either on the picosecond or the microsecond to millisecond timescale for the α2–α3 loop and α3 in ASC2 as was seen in NALP1.

It is to be noted that the model-free approach suffers from many complications that in principle could lead to erroneous results. The microdynamic parameters in the Lipari-Szabo (24) and the extended Lipari-Szabo (25) formalisms depend on the measured R_{1}, R_{2}, and (1H)H-15N NOE values in a highly non-linear fashion. Thus extraction of the microdynamic parameters relies on a highly non-linear, multidimensional optimization procedure that could involve as many as seven variables (26) potentially leading to an underdetermined system. Further, the correlation between local and global (μ) parameters complicates the error analysis (55). The presence of local dynamics on timescales approaching the overall correlation time (τ) leads to a breakdown of the Lipari-Szabo formalism and incorrect results. Finally, neglect of anisotropy in overall diffusion could also lead to erroneous microdynamic parameters. Thus, to corroborate our findings of a highly ordered system in the case of ASC2 (as opposed to the NALP1 PYD), we performed a reduced spectral density function analysis (Table 3 and Fig. 7). This approach suffers none of the complications of Lipari-Szabo approach being linearly dependent on the measured relaxation rates (27). The presence of extensive picosecond to nanosecond timescale dynamics leads to an increase in the value of the spectral density function at the 1H frequency. The values of J (0.87ω_{ij}) both at 600 MHz (6.56 ± 0.94 ps) and 800 MHz (3.18 ± 0.88 ps) were marginally lower for the helices (excluding α3) compared with the α2–α3 loop, including α3 (residues 28–45: 600 MHz, 8.21 ± 1.64 ps; 800 MHz, 3.77 ± 1.43 ps). These results, in agreement with the Lipari-Szabo analysis discussed above, indicated that the α2–α3 loop and the third helix were only marginally more disordered on the picosecond to nanosecond timescale than the other helical regions of ASC2. The regions that displayed the largest R_{ex} values in the model-free analysis above were also shown as containing R_{ex} contributions to their spin-spin relaxation rates in the reduced spectral density function analysis.

To further probe the presence of sub-millisecond timescale dynamics, we compared the R_{2} and R_{1p} at 600 MHz (Fig. 8). The R_{2}/R_{1p} ratio can be substantially greater than unity in the presence of a significant amount of sub-millisecond timescale
motion. In ASC2, the only residue that showed a statistically significant $R_2/R_1$ value was Asp-63 (1.14 ± 0.03) in the 4–5 loop. No sub-millisecond timescale motion was seen in the 2–3 loop or in the 3.

Surface Properties Suggest a Similarity with CARD-CARD Interactions—ASC2 is thought to antagonize inflammasome assembly and activity through homotypic PYD interactions with the adaptor protein ASC and/or NALP1 (2, 10), although the underlying mechanisms remain unclear. To date, only two distinct modes of interaction among DD superfamily members have been documented in detail, as exemplified by the crystal structures of complexes formed between the CARDs of Apaf-1 and caspase-9 (53) and the DDs of Tube and Pelle (56). The residues that form the interface are dramatically different between these two heterodimer structures, initially prompting suggestions that death motifs may associate by a variety of mechanisms (56). However, the view emerging from several recent studies is that there may be a limited number of ways for these homotypic interaction motifs to interact with each other and with other binding partners. For example, the DED of PEA-15 was found to use a similar motif to recognize its binding

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**TABLE 2**

Average backbone $^{15}$N relaxation rates for ASC2

<table>
<thead>
<tr>
<th></th>
<th>All (2–89)</th>
<th>Helices</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{1,600}$ (s$^{-1}$)</td>
<td>1.82 ± 0.08</td>
<td>1.84 ± 0.05</td>
</tr>
<tr>
<td>$R_{2,600}$ (s$^{-1}$)</td>
<td>9.02 ± 0.94</td>
<td>9.05 ± 0.49</td>
</tr>
<tr>
<td>NOE$_{600}$</td>
<td>0.74 ± 0.09</td>
<td>0.77 ± 0.04</td>
</tr>
<tr>
<td>$R_{1,800}$ (s$^{-1}$)</td>
<td>1.41 ± 0.06</td>
<td>1.42 ± 0.06</td>
</tr>
<tr>
<td>$R_{2,800}$ (s$^{-1}$)</td>
<td>10.48 ± 1.42</td>
<td>10.54 ± 0.88</td>
</tr>
<tr>
<td>NOE$_{800}$</td>
<td>0.84 ± 0.08</td>
<td>0.85 ± 0.04</td>
</tr>
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</table>

**FIGURE 5.** Relaxation rates of ASC2 measured at 600 and 800 MHz. NMR data for $R_1$ (A), $R_2$ (B), and (H$^\alpha$)$^{15}$N NOE (C). The errors signify the 68.3% confidence bounds ($\pm \sigma$, where $\sigma$ is the standard deviation for a one-parameter fit) obtained from the inverse covariance matrices of the respective fits.

**FIGURE 6.** Model-free analysis of ASC2 relaxation rates at 600 and 800 MHz. A, order parameter ($S^2$) values obtained from the analyses of the measured relaxation rates at 600 and 800 MHz using the Lipari-Szabo model-free formalism. B, $R_{ex}$ values obtained from the Lipari-Szabo model-free analyses. Only those residues with an $R_{ex}$ contribution $>15\%$ of the average $R_2$ rate at 600 MHz (>1.35 s$^{-1}$) are shown. The errors are indicated by the gray bars.

Surface Properties Suggest a Similarity with CARD-CARD Interactions—ASC2 is thought to antagonize inflammasome assembly and activity through homotypic PYD interactions with the adaptor protein ASC and/or NALP1 (2, 10), although the underlying mechanisms remain unclear. To date, only two distinct modes of interaction among DD superfamily members have been documented in detail, as exemplified by the crystal structures of complexes formed between the CARDs of Apaf-1 and caspase-9 (53) and the DDs of Tube and Pelle (56). The residues that form the interface are dramatically different between these two heterodimer structures, initially prompting suggestions that death motifs may associate by a variety of mechanisms (56). However, the view emerging from several recent studies is that there may be a limited number of ways for these homotypic interaction motifs to interact with each other and with other binding partners. For example, the DED of PEA-15 was found to use a similar motif to recognize its binding
partner as the Tube DD (57). In another study, the relative binding arrangement between the tandem DEDs of the viral caspase-8 inhibitory protein, MC159, has been demonstrated to closely resemble that between the CARDs of Apaf-1 and caspase-9 (58). Furthermore, it has been suggested that the adaptor protein FADD uses a similar binding mode to interact with caspase-8 as observed for the DED-DED association in MC159 (59). Thus, it appears that structurally conserved motifs may mediate interactions among DD superfamily members.

**TABLE 3**

Average dynamic parameters for ASC2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All (2–89)</th>
<th>Helices</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S^2$ (ns)</td>
<td>0.91 ± 0.08</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>$J(0)$ (ns)</td>
<td>2.28 ± 0.28</td>
<td>2.26 ± 0.22</td>
</tr>
<tr>
<td>$J'(0.87\omega_n)$ (ns)</td>
<td>0.34 ± 0.02</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>$J'(0.87\omega_n)$ (ps)</td>
<td>7.19 ± 2.03</td>
<td>6.69 ± 1.14</td>
</tr>
<tr>
<td>$J'(0.87\omega_n)$ (ps)</td>
<td>0.23 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>$J'(0.87\omega_n)$ (ps)</td>
<td>3.44 ± 1.58</td>
<td>3.25 ± 0.96</td>
</tr>
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</table>

It has also been postulated that PYD interactions may be analogous to those between the CARDs of Apaf-1 and caspase-9, with residues in helices 2 and 3 of one PYD forming contacts with helices 1 and 4 of a complementary PYD (19). However, at present there is no structural information or functional mutagenesis studies of a PYD heterodimer complex. Many charged residues are present on the surface of ASC2 (Fig. 9), suggesting that electrostatic interactions may play an important role in its association with ASC and NALP1. Although the surface patches of charged residues exhibit a balanced distribution of positive and negative potential, there is a distinct contiguous patch of negatively charged residues formed by residues in helices 1 and 4. Acidic amino acid residues that form this surface include Glu-13, Asp-48, Asp-51, and Asp-54. Another distinctive feature is a basic patch comprising residues Lys-21, Lys-22, Arg-38, and Arg-41 in helices 2 and 3. Interestingly, extensive alanine-scanning mutagenesis performed on ASC has identified several charged residues in analogous regions that play a crucial role in

**FIGURE 7.** Reduced spectral density function analysis of ASC2 relaxation rates at 600 and 800 MHz. A, the spectral density function at zero frequency $J(0)$ calculated using Equation 9 and relaxation data at 600 and 800 MHz. B, reduced spectral density function $J'(0.87\omega_n)$ at 800 (black) and 600 MHz (red). C, reduced spectral density function $J'(0.87\omega_n)$ at 800 (black) and 600 MHz (red). D, $R_m$ contributions to the $R_2$ rate at 600 MHz calculated using Equation 10. Only those residues with an $R_m$ contribution >15% of the average $R_2$ rate at 600 MHz (>1.35 s$^{-1}$) are shown. The errors are indicated by the red bars.
its ability to form cytoplasmic filaments through self-association (60). Mutations of residues Lys-21, Lys-26, Arg-41, Asp-48, and Asp-51 in helices 2, 3, and 4 were found to significantly compromise or abolish filament formation by the ASC PYD. These residues are highly conserved among human PYDs (60) and map primarily to the distinct charged patches identified on the surface of ASC2 (Fig. 9). This suggests that these surface patches are likely involved in the association of PYDs, but additional mutagenesis studies are required to elucidate precisely how PYDs interact.

DISCUSSION

Three-dimensional structure determination of the PYD-only protein ASC2 revealed a core structure of six antiparallel \( \alpha \)-helices. The structure of ASC2 closely resembles that of the ASC PYD (19), as expected from the high degree of sequence similarity, and the overall arrangement of the helices is comparable to other DD superfamily members. A prominent feature that distinguishes the PYD subfamily is the presence of an insertion between helices 2 and 3. In ASC and ASC2, helix 3 is preceded by a long loop of irregular structure. This loop is well defined by contacts with the remainder of the domain, however, both the corresponding region and helix 3 were found to be unstructured in the NALP1 PYD (20). These differences in conformation of helix 3 and the preceding loop may be linked to the amino acid composition and the absence of several hydrophobic residues in NALP1 (Fig. 4B). Further analysis of pyrin domain sequences revealed that the length and amino acid composition of the \( \alpha_2-\alpha_3 \) loop is highly variable (supplemental Fig. S1). Indeed, this loop is reduced or not present in several human PYDs, including the interferon-inducible AIM2, IFI16, and MNDA proteins, as well as the viral sequences. Thus, variations in the conformation or orientation of the \( \alpha_2-\alpha_3 \) loop may exist in different pyrin domains, possibly reflecting differences in their cellular function.

The emerging picture from structural studies of PYDs and other DD superfamily members, is a core fold of six \( \alpha \)-helices with the ability to accommodate significant structural variability. The most structurally diverse element of the fold is helix 3 and the preceding loop. This region can accommodate longer loops as seen in PYDs, or even additional helices as illustrated by the insertion of two short helices in the Tube DD (56). Conversely, helix 3 is reduced in length or not present at all in several of the known structures. Because helix 3 often protrudes prominently and does not contribute to the hydrophobic core, it can unfold locally without perturbing the remainder of the protein structure (20, 61). The third \( \alpha \)-helix can also be replaced by a short loop as observed in the crystal structure of MC159 (58, 62). Thus, in addition to an important role in protein-protein interactions (21, 53, 54), helix 3 is a hot spot for structural diversity in the DD superfamily.

The unexpected observation that helix 3 is flexibly disordered in NALP1 has led to speculation that PYD interactions may involve a conformational change upon binding. A detailed analysis of the conformational flexibility of ASC2 indicated that, although the \( \alpha_2-\alpha_3 \) loop and \( \alpha_3 \) were marginally more flexible than other helical regions of the protein, the complete disorder seen in the corresponding region of NALP1 was not present. This makes the NALP1 structure unique among PYDs of known structure. Thus, the suggestion that a coil to helix transition underlies protein-protein interactions involving PYDs (20, 22, 23) may not be as general as originally believed. Although the absence of flexibility does not preclude the possibility of conformational changes upon binding, a transition from complete disorder to order is likely to be unique to
NALP1 and not a general characteristic of pyrin domain interactions.

During the preparation of this report, the solution structure of ASC2 was also reported by Espejo and Patarroyo (63). Unexpectedly, this structure displayed striking differences from the structure of ASC2 described herein. Although the length of the α-helices is largely consistent with our studies, their relative orientations display substantial differences (supplemental Fig. S2). Most notable are the differences in the orientation of helices 2 and 4. In addition, the region corresponding to α2–α4 is unstructured, reminiscent of the NALP1 PYD (20). The disorder observed for this region is in marked contrast to our results, in which this structural element is well ordered due to the observation of numerous long range NOEs that unambiguously define its position. This is corroborated by the backbone relaxation data (Figs. 5–8), which indicate no dramatically increased mobility of residues in this region of the molecule.

The solution conditions used in the present study are essentially the same as those employed by Espejo and colleagues (52, 63), however, there were some minor differences in the ASC2 constructs studied. We employed an uncleaved N-terminal His6 tag, whereas Espejo and colleagues produced ASC2 as a glutathione S-transferase fusion protein that retained an extra 14 residues at the N terminus following cleavage (52, 63). Nevertheless, in both cases the additional N-terminal residues were unstructured and would not be expected to perturb the pyrin domain structure to any significant extent. The poor definition of the α2–α4 loop in the solution conditions used by Espejo and Patarroyo (63) may reflect a lack of NMR restraints in this region of the molecule due to errors in resonance assignments and in the interpretation of NOE data. We noted several discrepancies with the published resonance assignments (52), including a number of residues in the α2–α4 region (Thr-29, Val-30, Pro-40, and Arg-41) (supplemental Fig. S3). It is well established that even a small number of misinterpreted NOE signals can have dramatic consequences, ranging from a difference in orientation to a largely incorrect fold (64). For comparison, we also evaluated the agreement of the ASC2 structures determined by Espejo and Patarroyo (63) with the experimental 1DnH RDC data. The calculated RDC values are in poor agreement with the experimental data, with a correlation coefficient of 0.369 (supplemental Fig. S2D). The correlation in our case was 0.836 for an NOE-refined ensemble of structures prior to the inclusion of RDC constraints (Fig. 3C). A further indicator that this structure ensemble is erroneous is the low percentage of residues in the most favored regions of the Ramachandran plot (56%).

The analysis of the structure and backbone dynamics of ASC2 provided herein will provide useful information for further exploration of the function and interactions of PYDs. As modulators of caspase-1 activation represent attractive therapeutic targets, the structure of ASC2 represents a first step toward understanding the molecular basis for its inhibition of inflammasome activity.

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Aswin Natarajan, Ranajeet Ghose and Justine M. Hill

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