Solution Structure of the Tandem Src Homology 3 Domains of p47^{phox} in an Autoinhibited Form*

Satoru Yuzawa‡§§, Kenji Ogura‡§§, Masataka Horiuchi‡§§, Nobuo N. Suzuki‡§§, Yuko Fujioka‡§§, Mikio Kataoka¶, Hideki Sumimoto§§ and Fuyuhiko Inagaki‡§§

From the Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, CREST, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan, the Department of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara 630-0101, Japan, and the Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

The phagocyte NADPH oxidase is a multisubunit enzyme responsible for the generation of superoxide anions (O\textsuperscript{2−}) that kill invading microorganisms. p47\textsuperscript{phox} is a cytosolic subunit of the phagocyte NADPH oxidase, which plays a crucial role in the assembly of the activated NADPH oxidase complex. The molecular shapes of the p47\textsuperscript{phox} tandem SH3 domains either with or without a polybasic/autoinhibitory region (PBR/AIR) at the C terminus were studied using small angle x-ray scattering. The tandem SH3 domains with PBR/AIR formed a compact globular structure, whereas the tandem SH3 domains lacking the PBR/AIR formed an elongated structure. Alignment anisotropy analysis by NMR based on the residual dipolar couplings revealed that the tandem SH3 domains with PBR/AIR were in good agreement with a globular module corresponding to the split half of the intertwined dimer in crystalline state. The structure of the globular module was elucidated to represent a solution structure of the tandem SH3 domain in the autoinhibited form, where the PBR/AIR bundles the tandem SH3 domains and the linker forming a closed structure. Once PBR/AIR is released by phosphorylation, rearrangements of the SH3 domains may occur, forming an open structure that binds to the cytoplasmic proline-rich region of membrane-bound p22\textsuperscript{phox}.

The NADPH oxidase catalyzes the reduction of oxygen to a superoxide anion using NADPH as an electron donor, which plays a critical role in killing invading microorganisms in neutrophils and other phagocytic cells. The phagocyte NADPH oxidase comprises membrane-bound flavocytochrome b\textsubscript{558}, a heterodimer of gp91\textsuperscript{phox} and p22\textsuperscript{phox}, and at least four cytosolic regulatory subunits consisting of p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, and Rac (1–5). Upon activation of the cell, the p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox} complex translocates from the cytosol to the plasma membrane to associate with flavocytochrome b\textsubscript{558}, so that the NADPH oxidase subunits are assembled into the activated complex. The interaction between p47\textsuperscript{phox} and p22\textsuperscript{phox} is considered to be essential for NADPH oxidase activation (6–8).

The p47\textsuperscript{phox} subunit contains a phox homology domain, tandem SH3 domains, a polybasic region/autoinhibitory region (PBR/AIR), and a proline-rich region in this order (Fig. 1A). In resting cells, the tandem SH3 domains of p47\textsuperscript{phox} are masked through an intramolecular interaction with PBR/AIR, resulting in an autoinhibited form (6, 7, 9–11). Upon cell stimulation, a number of serine residues in PBR/AIR are phosphorylated (12–15). Phosphorylation of p47\textsuperscript{phox} induces conformational changes that subsequently lead to rearrangements in intramolecular interactions and the exposure of the tandem SH3 domains that enable interaction with the p22\textsuperscript{phox} subunit in flavocytochrome b\textsubscript{558} (6, 10, 11). Anionic amphiphiles such as arachidonate and SDS, activators of the NADPH oxidase in vitro, facilitate a conformational change of p47\textsuperscript{phox}, exposing its SH3 domains as well as inducing phosphorylation (6, 16, 17).

Recently, the structures of the autoinhibited and the activated forms of the tandem SH3 domains of p47\textsuperscript{phox} (Protein Data Bank codes 1NG2 and 1OV3) were reported (18). We also determined the structure of the tandem SH3 domains in the autoinhibited form (p47\textsuperscript{phox}(151–340); Protein Data Bank code 1UEC) independently (19, 20). The structure of the autoinhibited form of the tandem SH3 domains reveals that elongated monomers are related by a crystallographic 2-fold axis at the hinge, forming an intertwined dimer with a dumbbell-like shape (89 × 64 × 64 Å). The strand-exchanged region where βA, βB, and βC of the N-terminal moiety of one monomer were intertwined with βD, the 310 helical region, and βE of the other (Fig. 1, B and C) takes a typical SH3 fold. This strand-exchanged and intertwined region was identified as the N-terminal SH3 domain, although the distal loop of the canonical SH3 fold was extended to form the hinge. The split half of the intertwined dimer in the crystal structure has been assumed to be physiologically relevant and to represent the structure of the tandem SH3 domains in the autoinhibited form (18, 20), which we called the globular module (Fig. 1B).

However, a crucial issue remains to be clarified: whether the globular module exists in solution and if it represents the structure of the autoinhibited form of the tandem SH3 domains. This prompted us to investigate the structural characterization of the tandem SH3 domains in the autoinhibited form in solution. Herein, we report the structural properties of p47\textsuperscript{phox}(151–340) in solution elucidated by NMR spectroscopy and small angle x-ray scattering (SAXS) analysis.

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** To whom correspondence should be addressed. Tel.: 81-11-706-3975; Fax: 81-11-706-4979; E-mail: finagaki@pharm.hokudai.ac.jp.

† The abbreviations used are: SH3 domain, Src homology domain 3; PBR, polybasic region; AIR, autoinhibitory region; SAXS, small angle x-ray scattering; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser enhancement; RDC, residual dipolar coupling; IP, in-phase; AP, anti-phase; N-SH3, the N-terminal SH3 domain; C-SH3, C-terminal SH3 domain.

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**Structure of Autoinhibited Tandem SH3 Domains of p47phox**

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The truncated form of p47phox, residues 151–340 (p47phox(151–340)) including PBR/AIR, was expressed and purified as previously reported (19). The truncated form of p47phox, residues 151–286, corresponding to the tandem SH3 domains without PBR/AIR (p47phox(151–286)), was cloned into a pGEX-2T vector (Amersham Biosciences), transformed in Escherichia coli BL21(DE3), and expressed. The truncated form of p47phox, residues 151–286, was then removed by incubation with thrombin (151–340) (Pro -340, residues 151–340). The truncated form of p47phox, residues 151–340), was cloned into a pGEX-2T vector (Amersham Biosciences), transformed in Escherichia coli BL21(DE3), and expressed, as previously described (17). The cells were disrupted by sonication at 4 °C in 25 mM Tris-buffered saline buffer at pH 7.4. The protein was applied to a glutathione-Sepharose 4B column (Amersham Biosciences) equilibrated with phosphate-buffered saline buffer at pH 7.4, and the bound protein was eluted using 25 mM reduced glutathione. The N-terminal glutathione transferase tag of p47phox(151–286) was then removed by incubation with thrombin protease (Amersham Biosciences) for 12 h at 25 °C, and the digested protein was dialyzed against 2 liters of 25 mM Tris buffer at pH 8.0. The protein was purified by anion exchange chromatography on a Resource 6-ml column (Amersham Biosciences) and p47phox(151–340) was estimated in comparison with the standard curve.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were carried out using a Beckman Model XL-I analytical ultracentrifuge (Beckman Coulter, Inc.) equipped with both absorbance and interference optical detection system with an An-60 Ti rotor at 25 °C. Loaded p47phox(151–340) sample concentrations were 1.5, 2.8, and 4.2 mg/ml in 25 mM BisTris buffer at pH 6.5 and 150 mM NaCl. The protein samples were 110 µl with 130 µl of reference buffer in six-channel centerpiece. The data were collected at equilibrium for three different angular velocities: 12,000, 16,000, and 20,000 rpm. An interference fringe was measured using the Rayleigh interference optics. After 12 h of centrifugation, displacement of the interference fringes was compared at 2-h intervals to ensure that the sedimentation equilibrium was reached. Data analysis was carried out using the Beckman Optima XL-A/AXL-I software, version 4.1 (Beckman Coulter, Inc.) based on the Origin software (Microcal, Inc.).

**Small Angle X-ray Scattering**—SAXS data were collected on both p47phox(151–340) and p47phox(151–286) within a concentration range of...
the proteins from 2 to 16 mg/ml to estimate the possible effects of protein concentration on the determination of structural parameters. All of the measurements were made using a SAXS diffractometer in BL-10C installed at the Photon Factory in Tukuba, Japan (21, 22). The wavelength of the x-ray was 1.488 Å. The sample cell had a volume of 50 μl and a 1-mm path length with quartz windows. The data acquisition time was 600 s for each measurement. The identical buffer solution to the sample was recorded to measure solvent scattering. Protein scattering was obtained by subtracting the solvent scattering as the background trace.

The scattering data were analyzed using the Guinier approximation \( I(Q) = I(0) \exp(-R_g^2Q^2/2) \), where \( Q, R_g \) and \( I(0) \) were the momentum transfer, the radius of gyration, and the intensity at the zero scattering angle, respectively. \( I(0) \) was defined as \( I(0) = 4\pi \rho \alpha \frac{d\rho}{dV} \), where \( \alpha \) and \( \lambda \) were the scattering angle and wavelength of the x-rays, respectively. The \( I(0) \) and \( R_g \) values were calculated using the intensity of zero angle \((\text{ln}(I(0)))\) and slope \((-R_g^2/2\lambda)\) by linear extrapolation of the Guinier plots in the range of \( Q < R_g \times 1.8 \) (22–24). To estimate the relative molecular weight of scattering species, the zero angle intensity \( I(0) \) was scaled to the relative molecular weight using the scattering data for bovine carbonic anhydrase (Sigma-Aldrich), a monomeric protein with a molecular mass of 29 kDa.

The distance distribution function, \( R \), defined by \( P(r) = 1/2\pi \int |2\pi Q_r \sin(Q_r)| dQ_r \) corresponds to the distribution of distance, \( r \), between the volume element. \( P(r) \) was calculated by the indirect Fourier transform algorithm using the GNOM program (25). The \( Q \) ranges used in the \( P(r) \) analysis were from 0.02 to 0.30 Å⁻¹ for p47\textsuperscript{phox}(151–340) and from 0.03 to 0.19 Å⁻¹ for p47\textsuperscript{phox}(151–286). The \( R_g \) values, calculated as \( R_g^2 = \int r^2 P(r) dr/\int P(r) dr \), were estimated from the distance distribution function, \( P(r) \). The \( D_{\text{max}} \) was a maximum dimension. Structural parameters were derived using both Guinier analysis and the distance distribution function, \( P(r) \). The distance distribution functions using the crystal structure coordinates (Protein Data Bank code 1UEC) were estimated from the frequency of distances among carbon, nitrogen, and oxygen atoms in a 1-Å interval (24). The \( R \) was calculated from the coordinates. The maximum dimension, \( D_{\text{max}} \), was derived from the point at which \( P(r) \) approaches \( \nu_0 \), the maximum intensity.

Solution models of p47\textsuperscript{phox}(151–340) were generated from experimental scattering data by the ab initio shape determination program DAMMIN (26). DAMMIN calculates a volume of a protein filled with densely packed spheres (dummy atoms) to fit the experimental scattering data by a simulated annealing minimization procedure. The scattering data in the range of the momentum transfer with a \( Q \) value of 0.02–0.30 Å⁻¹ were used for the fit. Ten independent fits were run with DAMMIN. The independent models were superimposed using the program SUPCOMB (27) and averaged by the program DAMAVER (28), highlighting common structural features.

**NMR Spectroscopy**—The sequence specific resonance assignments of p47\textsuperscript{phox}(151–340) were previously described (29). A NMR sample containing 1.0 mM 15N-labeled p47\textsuperscript{phox}(151–340) in 25 mM BisTris buffer, 150 mM NaCl, pH 6.5, in 90% H₂O, 10% D₂O was utilized for the steady state heteronuclear 1H-15N NOE experiments. The NOE spectra were recorded on a Varian Unity plus 600 MHz NMR spectrometer at 25 °C using a saturation enhanced technique with pulsed field gradients (30). Four sets of the spectra were acquired using a 3.0 s relaxation delay in the experiment.

The 15N-labeled sample containing ~0.3 mM p47\textsuperscript{phox}(151–340) protein in 25 mM BisTris buffer (pH 6.5) and 150 mM NaCl was lyophilized from water and dissolved into 99.9% D₂O. After leaving the sample for ~15 min to allow temperature equilibration, 1H-15N HSQC spectra were recorded under identical conditions at different time intervals. The total measurement time for each HSQC spectrum was 30 min.

Two NMR samples were prepared to measure 1H-15N residual dipolar couplings in 50% of 90% H₂O/10% D₂O (pH 6.5) in Wilmad sample tubes, the isotropic sample, and the aligned sample. The isotropic sample contains 1 mM protein dissolved in 25 mM BisTris and 150 mM NaCl. The aligned sample contains 0.3 mM protein dissolved in isotropic sample buffer with an additional 5% C12E5 polyethylene glycol/\( \beta \)-octanol mixture with a molar ratio of surfactant to alcohol of 0.96 (31). The weak alignment was established throughout the measurements because the deuterium signal was observed as a sharp doublet with ~24 Hz splitting at 25 °C before and after the measurements. One-bond \( ^1H \)–15N coupling constants were measured on a Varian Unity INOVA 800 MHz NMR spectrometer at 25 °C using two-dimensional \( ^1H \)–15N IPAP-type sensitivity enhanced HSQC (IPAP HSQC) spectra with in-phase (IP) or anti-phase (AP) selections (32, 33). The weighted sum and difference of the IP and AP IPAP HSQC spectra yielded spectra displaying only the up-field and down-field \( ^1H \)–15N doublet components. The residual dipolar coupling (RDC) values were obtained by subtracting the observed coupling values of the isotropic sample from those of the aligned sample.

All of the two-dimensional NMR experiments were carried out using 256 and 1024 complex points in \( t_1 \) and \( t_2 \), respectively. Final data sets comprised 1024 and 4096 real points with a digital resolution of 2.9 and 2.7 Hz point in \( F_1 \) and \( F_2 \), respectively. All of the pulse sequences were a modified version of the Varian Protein Pack (www.varianinc.com).
NMR spectra were processed using VNMR (Varian Instruments, Palo Alto, CA), or NMRPipe (34). TALOS software was used to predict backbone dihedral angles (35). Fitting of the dipolar couplings to the structure was made using the Module program (36). All of the structure figures were prepared using PyMOL (37).

RESULTS AND DISCUSSION

Molecular Mass in Solution—Because the tandem SH3 domains in the autoinhibited form of p47phox (151–340) exist as an intertwined dimer in the crystalline state, we first investigated whether p47phox (151–340) could form a dimer or not in solution. Analytical size exclusion chromatography of purified p47phox (151–340) indicated that the protein forms a single species with no evidence of aggregation (Fig. 2A). Calibration with molecular weight standards revealed that p47phox (151–340) corresponded to a globular protein of ~24 kDa, roughly consistent with the molecular mass calculated by the primary amino acid sequence (~22 kDa).

The sedimentation equilibrium experiments were subsequently performed, which give a molecular weight independent of a molecular shape. The sedimentation equilibrium data for
the p47<sub>phox</sub>(151–340) at three different speeds are shown in Fig. 2B (lower panel). The data were analyzed based on the monomeric model. The goodness of fit to the model is excellent as shown in Fig. 2B (upper panel). We estimated an average molecular mass of 21400 ± 800 Da for p47<sub>phox</sub>(151–340), which is within an experimental error of the molecular mass of 21989 calculated for the p47<sub>phox</sub>(151–340) monomer from its amino acid sequence. A higher order oligomer or molecular aggregate was not detected.

**Molecular Mass and Average Size by Small Angle X-ray Scattering**—The molecular masses and average sizes of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) were estimated by SAXS analysis. The measured SAXS profiles showed obvious differences between p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) (Fig. 3A and 3B). Because the SAXS profile is sensitive to the size and shape of scattering molecules, the difference was attributed to the difference in the structures of the two proteins. A more quantitative representation of the structural difference can be obtained from the analyses of the Guinier approximation of the scattering data, which provides two structural parameters, the radius of gyration (R<sub>g</sub>) and the relative molecular mass (M<sub>r</sub>) from the zero angle scattering intensity, I(0). The small angle regions of the Guinier plots (lnI(Q) versus Q<sup>2</sup>) obtained from the scattering data were fitted to a single straight line (see “Experimental Procedures” for the definition of I(Q) and Q), as shown in Fig. 3 (C and D). These data sets for each protein were considered free of high molecular mass aggregates, because an upward shift in the lower angle region of the Guinier plots was not detected (22–24). Each of these data sets showed no concentration dependence in R<sub>g</sub> and I(0), indicating that no concentration dependent interaction existed. The relative molecular masses of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) were scaled using the scattering data for bovine carbonic anhydrase, a monomeric protein with a molecular mass of 29 kDa. The molecular masses of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) were estimated to be 22.5 and 15.4 kDa, respectively. These values are in good agreement with those calculated from the amino acid sequences. Considering the molecular mass estimated from the gel filtration analysis, the sedimentation equilibrium analysis, and the small angle x-ray scattering data, we concluded that both proteins exist as monomers in solution. Interestingly, R<sub>g</sub> of p47<sub>phox</sub>(151–340) was found to be 19.3 Å in contrast to 25.4 Å of p47<sub>phox</sub>(151–286), showing that the average molecular dimension of p47<sub>phox</sub>(151–340) is much smaller than that of p47<sub>phox</sub>(151–286) (Table I).

**Molecular Shapes and Dimensions of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) Estimated from the Distance Distribution Function**—The distance distribution function, P(r) reveals the approximate histogram for the interatomic distances between carbon, nitrogen, and oxygen atoms in a molecule and directly gives information on the shape of the molecules. Therefore, P(r) provides a quantitative evaluation of the conformational properties of proteins in solution. P(r) functions of p47<sub>phox</sub>(151–286) and p47<sub>phox</sub>(151–340) were evaluated from a set of scattering data using the indirect Fourier transform method (25). The observed structural parameters from the P(r) analysis are summarized in Table I together with those estimated from the Guinier approximation. The P(r) functions of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) are shown in Fig. 3 (E and F), respectively. The P(r) of p47<sub>phox</sub>(151–340) showed a single Gaussian-like curve with a peak at 22 Å and a half-width of 28 Å. Because

### Table 1

| Summary of structural parameters of p47<sub>phox</sub>(151–340) and p47<sub>phox</sub>(151–286) |
|-----------------------------------------------|
| p47<sub>phox</sub>(151–340) | p47<sub>phox</sub>(151–286) |
| Guinier approximation | | |
| R<sub>g</sub> (Å) | 19.3 (0.5) | 25.4 (0.9) |
| M<sub>r</sub> sas (kDa) | 22.5 (0.6) | 15.4 (0.6) |
| M<sub>r</sub> seq (kDa) | 22.0 | 15.3 |
| P(r) analysis | | |
| R<sub>g</sub> (Å) | 19.2 (0.3) | 25.3 (0.8) |
| D<sub>max</sub> (Å) | 60 | 80 |
| Ab initio shape analysis | | |
| R<sub>g</sub> (Å) | 19.32 (0.01) | | |
| D<sub>max</sub> (Å) | 59.3 (0.2) | | |
| χ<sup>2</sup> | 0.51 | | |

<sup>a</sup> R<sub>g</sub> is the radius of gyration, derived from the scattering data using Guinier approximation, the program GNOM, and the DAMMIN.

<sup>b</sup> The values in parentheses indicate standard deviations of the structural parameters estimated by SAXS analysis.

<sup>c</sup> M<sub>r</sub> sas and M<sub>r</sub> seq are molecular masses estimated from the scattering data and calculated from the primary sequence as a monomer.

<sup>d</sup> D<sub>max</sub> is a maximum dimension.

<sup>e</sup> Ab initio molecular shapes were calculated with the program DAMMIN.
a Gaussian-like curve is characteristic to a spherical molecule, p47phox(151–340) was considered a globular protein (Fig. 3E). Contrarily, the P(r) of p47phox(151–286) showed a curve with a peak located at nearly 25 Å, and the spread of the distribution curve extended to 80 Å (Fig. 3F), suggesting a relatively elongated structure. The Rg values for p47phox(151–340) and p47phox(151–286) were also estimated to be 19.2 and 25.3 Å, respectively, based on analyses of the P(r) functions in good agreement with those calculated from the Guinier approximation (Table I). Thus, we concluded that p47phox(151–340) takes a compact structure, in contrast to the extended structure of p47phox(151–286).

Ab Initio Shape Analysis of p47phox(151–340) by SAXS—The low resolution model of p47phox(151–340) was determined by ab initio molecular shape analysis using the simulated annealing program DAMMIN (26) based on the scattering data of p47phox(151–340). It is important to note that this shape analysis does not provide a unique solution but gives an ensemble of possible solutions consistent with the SAXS data. Ten independent runs of ab initio analysis were performed, all of which yielded similar results, considering the structural similarity of the calculated models. The models were superimposed, and an averaged model was constructed. Although all of the calculated models yielded nearly identical scattering curves, the best model provided a fit to the experimental data with χ^2 = 0.51 for p47phox(151–340), as shown in Fig. 4A. The averaged model shown in Fig. 4B roughly fits the globular module in the intertwined dimer of the crystal structure, with respect to the molecular dimension and molecular shape. The Rg and Dmax from ab initio analysis were in good agreement with those from the Guinier approximation and the distance distribution function analysis (Table I). Therefore, we concluded that p47phox(151–340) is monomeric in solution and takes a compact and globular structure, consistent with the globular module in the crystalline state.

Comparison of Molecular Shapes for the Tandem SH3 Domains with PBR/AIR in Solution and in Crystal—The P(r) functions for the intertwined dimer and the globular module were approximately calculated as the pairwise distance distribution between carbons, nitrogens, and oxygens based on the crystal structure (Protein Data Bank code 1UEC). The P(r) calculated for the intertwined dimer had two peaks at 19 and 55 Å, and the spread extended to more than 98 Å, whereas the P(r) calculated from the globular module showed a Gaussian-like curve with a single peak at 19 Å and a half-width of 24 Å, characteristic of a globular protein. Notably, the P(r) obtained from SAXS measurements for p47phox(151–340) was similar to the Gaussian with a single peak at 22 Å (Fig. 3E). Considering the extreme difference in P(r) functions calculated from the intertwined dimer and from the globular module, the SAXS data strongly supports the proposal that the molecular shape of p47phox(151–340) in aqueous solution was quite similar to the globular module. We concluded that the globular module represents the structure of p47phox(151–340) in aqueous solution. Furthermore, the intertwined dimer in the crystalline state was not physiologically relevant but could be stabilized in the crystal lattice. This notion was further confirmed by NMR analyses of p47phox(151–340).

Structural Properties for p47phox(151–340) by NMR—NMR experiments were performed to evaluate the solution structure of p47phox(151–340). The line width in the NMR spectrum suggested that p47phox(151–340) existed as a ~22-kDa monomer rather than a ~44-kDa dimer, which is consistent with the results of the analytical size exclusion chromatography, the sedimentation equilibrium analysis and the SAXS analysis. The backbone ^1H and ^15N resonances of p47phox(151–340) were assigned as previously reported (29) and were utilized as probes in the structural study of p47phox(151–340) by NMR. According to the crystal structure, p47phox(151–340) was divided into the following domains, N-SH3 domain (residues 159–212), the linker connecting the N-SH3 and C-SH3 domains (residues 213–228), the C-SH3 domain (residues 229–282), and PBR/AIR (residues 283–331) (Fig. 5A) (18, 20). Backbone dihedral angles for p47phox(151–340) were predicted using TALOS software (35). There is an appreciably good correlation between the backbone dihedral angles predicted by TALOS and those calculated from the globular module (Protein Data Bank code 1UEC), indicating that the overall structure in solution is similar to the globular module. In an effort to investigate the stabilities of the secondary structural elements of p47phox(151–340) in solution, hydrogen-deuterium exchange measurements were applied using NMR. The ^1H–^15N HSQC spectra for monitoring the hydrogen-deuterium exchange rate of each amide proton enabled us to identify 63 amide protons with slow hydrogen-deuterium exchange rates, which were derived from the stable secondary structural elements. These amide protons are located in both SH3 domains, whereas all of the amide protons in the linker and PBR/AIR in the autoinhibited form were completely exchanged with solvent deuterons before the NMR measurements started.

Backbone Dynamics of p47phox(151–340)—In an effort to characterize the dynamics of each ^1H–^15N bond vector of p47phox(151–340) in solution, the steady state heteronuclear ^1H–^15N NOE was measured using the uniformly ^15N-labeled protein. p47phox(151–340) contains 10 proline residues. In addition, five residues, including 153, 189, 288, 324, and 326, overlapped considerably and were therefore excluded from NOE analysis. The 18 arginine residues are located distinctively in PBR/AIR of p47phox(151–340), thus specifically ^15N-labeled protein at arginine residues was prepared to investigate the dynamic behavior of PBR/AIR. Fig. 5B
summarizes a plot of the NOE values for each amino acid residue, where those of the arginine residues are indicated with blue bars.

The residues with large NOE values (0.7–1.0) were expected to have lower flexibility and to be located in the rigid core, whereas those with small or negative NOE values (<0.6) were expected to be located at the loop or linker region with higher flexibility (24, 38). The present measurements showed that N-SH3 (159–212), C-SH3 (229–282), and the residues 298–330 of PBR/AIR had large, positive NOE values (more than 0.7), whereas those with small or negative NOE values (<0.6) were expected to be located at the loop or linker region with higher flexibility (24, 38). The present measurements showed that N-SH3 (159–212), C-SH3 (229–282), and the residues 298–330 of PBR/AIR had large, positive NOE values (more than 0.7),

FIG. 6. Characterization of the alignment tensor for p47\textsuperscript{phox}(151–340). A and B, superposition of selected regions from the two-dimensional \textsuperscript{1}H–\textsuperscript{15}N IP-AP HSQC spectra of p47\textsuperscript{phox}(151–340). The marked splitting in the isotropic spectrum (A) corresponds to \textsuperscript{1}J_{NN}, and the marked splitting in the partially aligned spectrum (B) corresponds to the sum of \textsuperscript{1}J_{NN} and the residual dipolar coupling. \textsuperscript{1}H–\textsuperscript{15}N RDCs were determined by subtracting the splittings in the isotropic spectrum from those in the partially aligned spectrum. C and D, correlations between experimentally measured and the back-calculated \textsuperscript{1}H–\textsuperscript{15}N RDC values using the globular module from the intertwined dimer (Protein Data Bank code 1UEC) of p47\textsuperscript{phox}(151–340). The correlations between the calculated and the observed RDCs are shown for each SH3 domain (N-SH3 in blue and C-SH3 in green) (C) and for the entire molecule (D) of p47\textsuperscript{phox}(151–340). E and F, comparison of the alignment tensor between the individual domains (N-SH3 and C-SH3) (E) and the entire molecule (F) of p47\textsuperscript{phox}(151–340). The principal axes of the alignment tensors are indicated in red positioned at the center of mass of each SH3 domain (E) and the entire molecule (F). The principal axis of the inertia of the globular module is indicated in blue positioned at its center of mass. The SH3 domains are colored blue (N-SH3) and green (C-SH3), respectively (E), and the entire molecule is colored green (F).
demonstrating that in solution both SH3 domains and PBR/AIR (298–330) behaved as a folded structural core. In addition, the large NOE values were observed for linker residues 213–228, indicating that the linker was also involved in the structural core. However, the N-terminal region of PBR/AIR (residues 286–295) had slightly smaller NOE values (−0.3–0.6), indicating that this region was flexible. The N-terminal and C-terminal regions displayed smaller positive (<0.6) or negative NOE values, showing that these regions were flexible. In summary, the region of residues 159–333 of p47phox (151–340) comprising two SH3 domains, the linker, and PBR/AIR was a single folded unit in solution.

The Orientation of the SH3 Domains in the Autoinhibited Form Delineated by the Analysis of Residual Dipolar Couplings—In liquid crystalline media, a weak molecular alignment was induced by a static magnetic field, resulting in 1H–15N RDCs in the backbone amide groups (39). RDC is a sensitive probe for the structure in solution and has been utilized as a direct and simple tool to evaluate the consistency between solution structures and crystal structures. Recently, the analysis of the alignment tensor from RDC values has been successfully applied in an effort to determine the relative domain orientations of multidomain proteins (40–44).

The globular module in the crystal structure was investigated by analysis of the alignment tensor for the RDC values of p47phox (151–340) to determine whether it represents the solution structure. The highly flexible residues based on NOE experiments (NOE values < 0.65) were excluded from the analysis, including residues 150–159 (the N-terminal region); 199–201 (the distal loop in N-SH3); 269–271 (the distal loop in C-SH3); 284–297, 307–308, and 314 (PBR/AIR); and 334–340 (the C-terminal region). The residues involved in the structural core were subsequently applied to the RDC analysis. The RDC values for 126 residues (40 residues for N-SH3, 46 residues for C-SH3, and 40 residues for the linker and PBR/AIR) were obtained by subtracting the coupling values of the isotropic sample (Fig. 6A) from those of the aligned sample (Fig. 6B), which ranged from −33 to 37 Hz. The experimentally measured RDCs of p47phox (151–340) were then analyzed in an effort to search for the principal axes and the principal values of the alignment tensors.

The relative orientation of the two SH3 domains in solution might be different from that in the globular module because of the strand-exchanged dimer formation in crystal (Fig. 1, B and C), therefore each SH3 domain was assumed to have an individual alignment tensor. The alignment tensor and the RDC values, back-calculated from the structure of each SH3 domain, were analyzed to fit the observed RDC values. The error function, γ2, the measure of the agreement between the RDCs from the NMR experiments and those calculated from the model, were minimized to find the alignment tensor that fit the experimental data. The correlation between the experimental and the back-calculated RDC values for each SH3 domain is shown in Fig. 6C. Strong correlations between the experimental and the back-calculated RDCs for both the N-SH3 and C-SH3 domains were obtained. In addition, a close similarity between both alignment tensors was noticeable (Fig. 6, C and E, and Table II). These results indicate that the two SH3 domains have a common alignment tensor.

The single alignment tensor of p47phox (151–340) was then searched, and the back-calculated RDC values were compared with the experimental RDC values for the globular module, as shown in Fig. 6D. The principal axes of the alignment tensor and the tensor parameters are also shown in Fig. 6F and Table II. Low γ2 values were obtained using the structure of the globular module including a linker and PBR/AIR. The experimental RDC values are in good agreement with the back-calculated RDCs, with a correlation coefficient of 0.967 for the globular module (Fig. 6D). The globular module was determined to represent a solution structure in which RDC values could be explained by a single alignment tensor.

| Chain | Aa | η | α | β | γ | N | R<sub>ip</sub> | R<sub>0.65</sub> |
|-------|----|---|---|---|---|---|----------|----------|
| N-SH3 | 16.0 | 0.38 | 136 | 146 | −155 | 40 | 0.15 | 0.986 |
| C-SH3 | 16.0 | 0.26 | 149 | 145 | −153 | 46 | 0.18 | 0.980 |
| Globular module | 15.9 | 0.31 | 141 | 144 | −156 | 126 | 0.21 | 0.967 |

The dipolar coupling R factor (R<sub>ip</sub>) was used to access the quality of the derived alignment tensor (47, 48). The validity of the structural data obtained here is justified by the low values of R<sub>ip</sub>, indicating good agreement between the measured RDC values and the calculated RDCs from the structure.

The Euler angles, α, β, and γ define the rotation that transforms the molecular frame into the principal tensor frame.

* N is number of residues.
* The dipolar coupling R factor (R<sub>0.65</sub>) was used to access the quality of the derived alignment tensor (47, 48). The validity of the structural data obtained here is justified by the low values of R<sub>0.65</sub>, indicating good agreement between the measured RDC values and the calculated RDCs from the structure.

Table II: Characteristics of the molecular alignment tensor for N- and C-terminal SH3 domains in p47phox (151–340) at pH 6.5 in the ordered phase of C12E5 polyethylene glycol/n-hexanol.

The dipolar coupling ε factor (ε<sub>ip</sub>) was used to access the quality of the derived alignment tensor (47, 48). The validity of the structural data obtained here is justified by the low values of ε<sub>ip</sub>, indicating good agreement between the measured RDC values and the calculated RDCs from the structure.

Table II: Characteristics of the molecular alignment tensor for N- and C-terminal SH3 domains in p47phox (151–340) at pH 6.5 in the ordered phase of C12E5 polyethylene glycol/n-hexanol.
Structure of Autoinhibited Tandem SH3 Domains of p47phox

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Solution Structure of the Tandem Src Homology 3 Domains of p47phox in an Autoinhibited Form
Satoru Yuzawa, Kenji Ogura, Masataka Horiuchi, Nobuo N. Suzuki, Yuko Fujioka, Mikio Kataoka, Hideki Sumimoto and Fuyuhiko Inagaki

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