Hydrodynamic Studies on the Quaternary Structure of Recombinant Mouse Purβ*

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Purβ is a gene regulatory factor belonging to a family of highly conserved nucleic acid-binding proteins related by their ability to preferentially bind single-stranded DNA or RNA sequences rich in purine nucleotides. In conjunction with Purα, Purβ has been implicated in transcripational and translational repression of genes encoding contractile proteins found in the heart and vasculature. Although several models of sequence–specific DNA recognition, strand separation, and activator inhibition by oligomeric Purα and Purβ have been proposed, it is currently unclear whether protein–protein interaction is a prerequisite to, or a consequence of nucleic acid binding. In this study, a recombinant protein purification scheme was devised to yield homogenous mouse Purβ devoid of nucleic acid. Recombinant Purβ was then subjected to light scattering and analytical ultracentrifugation analyses to assess the size, shape, and oligomeric state of the purified protein in solution. Results of laser light scattering and sedimentation velocity experiments indicated that Purβ reversibly self-associates in the absence of nucleic acid. Both approaches independently showed that the hydrodynamic shape of the Purβ homodimer is markedly asymmetric and non-spherical. Sedimentation velocity analyses indicated that dimeric Purβ has a sedimentation coefficient of 3.96 Svedberg, a frictional coefficient ratio (f/f₀) of 1.60, and a hydrodynamic radius of 4.43 nm. These values were consistent with those determined by independent dynamic light scattering studies. Sedimentation equilibrium analyses confirmed that Purβ self-associates in a reversible monomer–dimer equilibrium characterized by a Kₐ = 1.13 ± 0.27 μM.

Purα and Purβ are members of a highly conserved family of nucleic acid-binding proteins related by primary structure and a propensity to interact with single-stranded DNA (ssDNA)2 or RNA sequences rich in purine nucleotides (for review, see Ref. 1). The founding member of this family, Purα, was initially identified by virtue of its ability to bind in a strand-specific manner to (GGN)n repeat-containing sequences (so called PUR elements) commonly found in gene flanking regions and in or near origins of DNA replication (2, 3). Accordingly, Purα has been implicated in regulating gene transcription, cell growth, and cell cycle progression (4–12). Purβ was similarly identified based on PUR element-dependent expression cloning using regulatory ssDNA sequences derived from human c-myec (2) and mouse smooth muscle α-actin (SMαA) genes (13). Like Purα, Purβ has also been reported to function as a transcription factor and, in the case of genes encoding SMαA and α-myosin heavy chain, to act in concert with Purα to repress gene expression at the level of transcription and translation (14, 15). Hence, in addition to their ability to bind ssDNA in a sequence–specific manner, interaction with RNA appears to be a critical biochemical property of the Pur family of proteins for modulation of mRNA translation and transport (16–19).

Purα and Purβ are ~70% identical at the amino acid level (13). Biochemical investigation of deletion mutants has shown that each protein possesses a minimal ssDNA/RNA binding domain composed of a unique set of highly homologous sequence repeats (4, 20, 21). The most significant sequence differences between Purα and Purβ exist near the N and C termini, suggesting that each protein may have evolved to perform distinct functions (13). Purα and Purβ have been reported to bind to a PUR element in a highly asymmetric polypurine/polypyrimidine tract located in the 5′-flanking region of the mouse SMαA gene (22, 23). It has been hypothesized that strand-specific binding by Purα/Purβ to this element disrupts a core MCAT enhancer motif, thereby repressing SMαA promoter activity in cultured fibroblasts and vascular smooth muscle cells (23). That Purα and Purβ function as inhibitors of SMαA expression is of particular interest due to the essential role played by SMαA in vascular contraction (24), cell motility (25), wound repair (26), and arterial remodeling (27, 28). Despite biochemical similarities, gain-of-function studies suggest that Purα and Purβ are not redundant in terms of their transcriptional repressor activity toward the full-length mouse SMαA promoter in transfected vascular smooth muscle cells (21, 29). In light of the specific protein–DNA, protein–RNA, and protein–protein interactions attributed to Purα and Purβ and their potential relevance in modulating cell growth and differentiation, a need has emerged for the elucidation of the biophysical factors governing nucleic acid recognition. The mechanism of

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§ The abbreviations used are: ssDNA, single-stranded DNA; SMαA, smooth muscle α-actin; SEC, size exclusion chromatography; LLS, laser light scattering; DLS, dynamic light scattering; RI, refractive index; Ni-NTA, nickel-nitrilotriacetic acid; Dᵥ, translational diffusion coefficient.
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DNA binding is of particular interest since DNA is thought to exist primarily in a double-stranded B-form helical configuration in vivo. Because Purα and Purβ preferentially bind to ssDNA or, in some cases, stem-loop (non-B-form) structures (30), this aspect of Pur protein function has been investigated to a greater degree. Several reports have indicated that Purα is capable of helix destabilization despite showing no intrinsic helicase or ATPase activity (31, 32). This has led to speculation that Pur proteins may promote strand displacement by opportunistic binding to transiently formed ssDNA structures. This notion is supported by the finding that the 5′-MCAT enhancer region of the SMαA promoter has the propensity to adopt a partially unpaired configuration in response to transforming growth factor β1 signaling (33). Another potentially important aspect of DNA binding that was hinted at by previous studies involves the oligomeric state of Pur proteins in the presence and absence of ssDNA. Experiments reported by Kelm et al. (22) suggested that Purα and Purβ bound to a SMαA promoter-derived PUR element as either hetero- or homodimeric nucleoprotein complexes. However, the conditions used in those experiments did not permit determination of whether or not dimerization was a prerequisite for, or a consequence of ssDNA binding. In this regard, a report by Gallia et al. (34) has pointed to a critical role for RNA in mediating self-association of Purα.

In the present study we elucidate the quaternary structure of purified recombinant Purβ by employing hydrodynamic and thermodynamic approaches to examine the macromolecular character of nucleic-acid free Purβ in solution. Size exclusion chromatography coupled to static and dynamic light scattering-based detection systems revealed Purβ to be an asymmetric protein capable of homodimeric self-association. This principal finding was confirmed by analytical ultracentrifugation which established that mouse Purβ does indeed exist in a reversible monomer-dimer equilibrium characterized by a dissociation constant of ~1 μM in the absence of nucleic acid. Hydrodynamic analyses further suggested that homodimeric Purβ assumes a non-spherical conformation in solution. We propose a model in which dimerization may affect ssDNA binding in a manner regulated by a mass action-governed self-association of Purβ. This type of a mechanism may be particularly relevant to pathophysiological states of the heart and vasculature where elevated Purβ levels have been noted (15, 35).

EXPERIMENTAL PROCEDURES

Purification of Nucleic Acid-free N-HisPurβ—Expression of N-terminal hexahistidine-tagged mouse Purβ (N-HisPurβ) in Escherichia coli (22, 29) was performed with modifications described in the online data supplement. Cell pellets equivalent to 4 liters were allowed to thaw in 20 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol) and completely resuspended on ice. Protease inhibitors leupeptin, aprotinin, and pepstatin A were each added to a final concentration of 1 μg/ml, and phenylmethylsulfonyl fluoride was added to 0.1 mM. Lysis was facilitated by the addition of egg white lysozyme (Sigma) to a final concentration of 1 mg/ml and incubation on ice for 20 min with occasional stirring. The cell suspension was sonicated with a Branson Sonifier model 150 (setting 10) for a total of six 10-s bursts with 1-min incubations on ice between bursts. Lysate was cleared by centrifugation at 14,000 × g for 30 min at 4 °C. A total of 5 ml of 50% Ni-NTA-agarose slurry (Qiagen) was added to the cleared lysate followed by 5 ml of NaCl to a final concentration of 1 M. Bovine pancreatic DNase I and RNase A (Sigma) were each added to 59 and 92 units/ml, respectively (based on manufacturer specified activities), and the lysate-Ni-NTA-agarose mixture was slowly rocked for 2 h at room temperature. The lysate-resin mixture was then gently centrifuged at 1000 × g for 2 min at 4 °C to pellet the Ni-NTA-agarose. Supernatant was removed, and 25 ml of buffer B (50 mM sodium phosphate, pH 8.0, 2 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol plus protease inhibitors) was added to the resin. The mixture was then incubated ~14 h at 4 °C with slow rocking. Gentle centrifugation at 1000 × g for 2 min at 4 °C was used to pellet the resin, which was subsequently resuspended in buffer A and loaded into a 1.5-cm diameter column. The rest of the purification procedure was carried out at room temperature. The resin was washed with buffer A until the absorbance of the flow through at 280 nm reached a base-line level (A280 = 0.02). N-HisPurβ was eluted by application of buffer C (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 500 mM imidazole, 10 mM β-mercaptoethanol). Eluted protein was concentrated using a centrifugal filter device (Centriprep YM-10, Millipore). Size exclusion chromatography was carried out on a 1.5 × 98-cm column packed with Sephacryl® 200 HR resin (Sigma) equilibrated in buffer E (50 mM sodium phosphate, pH 7.5, 2.00 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol) and run at a flow rate of 0.5 ml/min. The optical density of the eluate was monitored with a GE Healthcare model UV-1 UV-visible detector. The column was calibrated using bovine serum albumin, ovalbumin, carbonic anhydride, and cytochrome c protein standards (Sigma). Fractions corresponding to the major peak of dimeric N-HisPurβ were pooled and concentrated as described above.

Quality Control Measures and Quantification of N-HisPurβ—Relative purity of N-HisPurβ was assessed by Coomassie Blue staining of protein reduced with 300 mM β-mercaptoethanol and resolved by SDS-PAGE on 12% (40:1 acrylamide:bisacrylamide) mini-gels. Preparations used for hydrodynamic studies were judged to be >95% homogenous under reducing conditions. To assess the level of nucleic acid contamination, base-line-corrected absorbance spectra of purified N-HisPurβ were obtained using a Cary Bio100 dual beam spectrophotometer (Varian). A theoretical molar extinction spectrum of N-HisPurβ was calculated using SEDNTERP software (36) based on the method of Pace et al. (37). Protein concentration was determined spectrophotometrically assuming an extinction coefficient of 18,610 M−1 cm−1 at a wavelength of 280 nm and a monomeric relative molecular weight of 35,168.6 (calculated using SEDNTERP). As an extra quality control measure, purified N-HisPurβ (1.4 mg in 500 μl), was extracted twice with an equal volume of buffered phenol followed by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and an equal volume of chloroform. The aqueous phase of the extract was lyophilized and redissolved in ultrapure water 3 times and then dissolved one final time in 200 μl of ultra pure water. A base-line-corrected absorbance spec-
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trum of the lyophilized extract was obtained to ensure the absence of a peak at 260 nm.

Light Scattering Analysis of N-HisPurβ—Molecular mass and hydrodynamic radius measurements of N-HisPurβ in solution were made by size-exclusion chromatography (SEC)-coupled light scattering techniques. These experiments were performed at the W. M. Keck Foundation Biotechnology Resource Laboratory, Biophysics Facility, Yale University, New Haven, CT as described (38, 39). A comprehensive description of the experimental system and methods used for analysis of laser light scattering (LLS) and dynamic light scattering (DLS) data are provided in the online data supplement.

Sedimentation Velocity Analysis of N-HisPurβ—Sedimentation velocity experiments were carried out in a Beckman/Coulter Optima XL-I/XL-A analytical ultracentrifuge equipped with an An50Ti rotor. Radial concentration distributions were measured at 50,000 rpm and 4 °C using Rayleigh interference optics. Protein samples were gel filtered over a 1.5 × 98-cm Sephacryl® 200 HR size-exclusion column pre-equilibrated and eluted with buffer E as a final step before sedimentation. The fraction corresponding to the absorbance maximum of the presumed dimeric peak was used to prepare a dilution series of N-HisPurβ over a 10-fold concentration range starting at 43.5 μM. The reference buffer consisted of a buffer E eluate from the size exclusion column. Blank-subtracted Rayleigh interference scans were recorded at 1-min intervals. Sedimentation velocity data were analyzed by the dc/dt method to generate apparent sedimentation coefficient distributions, g(s*) (40), with the use of DCDT+ software (41). Direct fitting of time-resolved concentration difference curves (Δc versus radius) to numerical solutions of the Lamm equation describing multiple, interacting species models and kinetic models was performed using SEDANAL v4.3 software (42). Temperature-corrected values for the partial specific volume of N-HisPurβ (ν) as well as density (ρ) and viscosity (η) of buffer E were calculated using the program SEDNTERP (36). Resulting values are as follows: ρ = 0.7109 ml g⁻¹, ρ = 1.0149 g ml⁻¹, and η = 0.1635 g cm⁻¹ s⁻¹. Molecular shape modeling was also carried out with SEDNTERP. Protein integrity was assessed after sedimentation by SDS-PAGE to ensure that the samples were intact.

Sedimentation Equilibrium Analysis of N-HisPurβ—Sedimentation equilibrium experiments were carried out in cells fitted with six-sector charcoal-Epon centerpieces (1.2-cm path). Protein and reference buffer samples were prepared as described above. A dilution series of N-HisPurβ was made from the dimeric peak fraction off SEC over a 10-fold concentration range. Sedimentation was carried out at rotor speeds of 22,000, 28,000, and 35,000 rpm at 4 °C. Five scans were averaged to remove noise. Equilibrium was judged to be achieved by the superposition of scans taken 6 h apart and by analysis with the MATCH package implemented by HETEROANALYSIS software (43). To determine the stoichiometry of the reaction and the associated equilibrium constant, blank corrected sedimentation equilibrium data were fit using the HETEROANALYSIS software to expansions of the general Equation 1.

\[
A(r, \lambda) = \sum_i \epsilon_i \lambda C_i \exp[M_i \phi(r^2 - r_0^2)] 
\]

(Eq. 1)

Equation 1 represents a summation over all species i, where \(A(r, \lambda)\) is the radius-dependent absorbance at radial position \(r\) and wavelength \(\lambda\), \(\epsilon_i\) is the molar extinction coefficient, \(\lambda\) is the path length, \(C_i\) is the molar concentration of the ith species at the arbitrary reference radial position \(r_0\), \(M_i\) refers to the buoyant molecular weight of the ith species equal to \(M_i(1 - \vec{v}_i/\rho)\), where \(M_i\) is the relative molecular weight, \(\vec{v}_i\) is the partial specific volume, and \(\rho\) is the solvent density. \(\phi\) is equal to \(\omega^2/RT\), in which \(\omega\) is the angular velocity, \(R\) is the gas constant (8.314 × 10⁹ erg/mol K), and \(T\) is the absolute temperature. Model-specific expansions of Equation 1 included single ideal species, single non-ideal species, monomer-N-mer equilibria, monomer-dimer with incompetent monomer, monomer-dimer with incompetent dimer, and monomer-N-mer-Q-mer equilibria. Model-specific equations are listed as supplemental material.

RESULTS

Purification of Nucleic Acid-free Recombinant Purβ—To investigate the self-association of N-HisPurβ it was essential to ensure that preparations of the recombinant protein be devoid of co-purifying DNA and/or RNA. To accomplish this task we developed a protocol that included nucleases during E. coli lysis and performed Ni-NTA-agarose affinity chromatography under high ionic strength conditions to promote removal of weakly associated nucleic acids. A final SEC step was also included to eliminate high molecular weight aggregates and low molecular weight fragments. Using the method described herein, N-HisPurβ was purified to homogeneity as judged by SDS-PAGE (Fig. 1A). Although the molecular weight of N-HisPurβ calculated on the basis of its amino acid sequence is 35,168.6, it appears to migrate by SDS-PAGE as an ~43-kDa peptide under reducing conditions. The unusual electrophoretic mobility of the recombinant protein is consistent with the reported mobility of native Purβ expressed in fibroblasts and vascular smooth muscle cells (13, 23). This suggests that the His tag is not the major contributing factor to the non-ideal electrophoretic behavior of N-HisPurβ. To assess the extent of nucleic acid contamination, we compared the absorbance spec-
trum (normalized to the molar extinction at 280 nm of 18,610 m$^{-1}$ cm$^{-1}$) to a hypothetical molar extinction spectrum of N-HisPur$\beta$, generated from amino acid content (Fig. 1B). This comparison showed only minimal deviations between the calculated and experimental spectra in the region around 260 nm. It would be predicted that stoichiometric quantities of co-purifying nucleic acids would result in a large spectral difference in this range since nucleoside-5'-monophosphates have a molar extinction coefficient on the average of 10$^4$ M$^{-1}$ cm$^{-1}$ at or near 260 nm (44). Furthermore, absorbance spectra of phenol-chloroform extracts of purified N-HisPur$\beta$ preparations showed no species with a $\lambda_{\text{max}}$ of 260 nm (data not shown). Collectively, these data indicate that the preparations of N-HisPur$\beta$ used in this study were free of co-purifying nucleic acids.

Based on information obtained while optimizing the purification protocol, buffer E (see "Experimental Procedures") was chosen as the buffer condition for all sedimentation and light-scattering experiments. We have determined that the solubility of N-HisPur$\beta$ relies heavily on ionic strength and reducing agent concentration (data not shown). Dialysis of protein at moderate concentrations (~1 mg/ml) and low salt conditions leads to loss of protein (likely deposition on vessel surface, as sample can be recovered by the addition of high salt buffers). The salt concentration of buffer E (200 mM NaCl) was determined to be the minimum quantity to limit this effect and to maximize N-HisPur$\beta$ stability.

**Hydrodynamic Analysis of Recombinant Pur$\beta$ by Size Exclusion Chromatography-coupled Light Scattering Techniques**—A series of physical techniques that make use of macromolecular light scattering phenomena were used to investigate the hydrodynamic properties of N-HisPur$\beta$ in solution. SEC-LLS-DLS is a well suited means of investigating the hydrodynamic characteristics of proteins. It is non-destructive, and each individual light scattering detection technique can be performed in series after a size fractionation step. The use of SEC as a preliminary step to light-scattering can eliminate some of the ambiguity created in performing weight-average measurements (38, 39, 45).

The results of applying the SEC-LLS to solutions of N-HisPur$\beta$ reveal an asymmetric distribution of refractive index (RI) and molar mass measurements in the sole eluting peak of N-HisPur$\beta$, as compared with a bovine serum albumin standard (Fig. 2A). These results are consistent with a polydisperse mixture that elutes as a single, albeit asymmetric peak. On the sole basis of RI signal, one might surmise that the asymmetric shape of the elution peak could arise from interaction of N-HisPur$\beta$ with the column matrix. Although this could conceivably cause a tailing effect on elution, it would not cause a broadening in the distribution of weight-average molar mass measurements as is seen for N-HisPur$\beta$ (Fig. 2A). Instead, this effect is likely due to polydispersity. The number average of the weight average molar mass measurements across the elution peak is 67.7 ± 4.12 kDa, corresponding to a 6.08% degree of polydispersity. Based on the number average molar mass, this suggests that N-HisPur$\beta$ exists as an interacting mixture of monomers and dimers (expected dimer $M_\text{D}$ of 70,337.2).

Weight average hydrodynamic radii ($R_h$) determinations on SEC fractionated N-HisPur$\beta$ by DLS were also consistent with a self-associating system. It was found that the number average Weight average hydrodynamic radii ($R_h$) determinations on SEC fractionated N-HisPur$\beta$ by DLS were also consistent with a self-associating system. It was found that the number average $R_{hy}$ across the top 10% of the eluting protein peak (region of peak where scatter signal is strongest and $R_{hy}$ values are approximately constant) is dependent upon the loading quantity of N-HisPur$\beta$ (Fig. 2B). The resultant $R_{hy}$ values for loading quantities of 19.9 and 5.1 pmol were 4.3 and 3.8 nm, respectively. From these data, it is apparent that the weight average $R_{hy}$ of the eluting protein is dependent upon a mass-action-governed self-association of N-HisPur$\beta$. These $R_{hy}$ values are very different from the calculated $R_{hy} = 2.72$ nm for an equivalent non-compressible sphere with a molecular mass of 70,337.3 Da. The $R_{hy}$ value of 4.3 nm is expected to result in a frictional coefficient ratio ($f/f_0$) of 1.56, as determined by analysis with Dynamics Software (see the supplemental methods). Collectively, findings based on light scattering suggest that N-HisPur$\beta$ participates in a monomer-dimer equilibrium and that the dimeric form assumes an asymmetric shape in solution.

**Sedimentation Velocity Analysis of Recombinant Pur$\beta$—Quantitative hydrodynamic and thermodynamic analyses aimed at...**
investigating the oligomeric structure of N-HisPurβ in solution were performed by analytical ultracentrifugation. Sedimentation velocity experiments were carried out to investigate the hydrodynamic properties of recombinant Purβ and to validate the observations made by SEC-LLS-DLS studies. The apparent sedimentation coefficient distribution function, $g(s^*)$, for solutions of N-HisPurβ at four loading concentrations ranging from 4.7 to 43.5 μM is shown in Fig. 3A. Analysis of the normalized weight average sedimentation coefficient ($s_{20w}$) from $g(s^*)$ distributions shows an increase as a function of loading concentration (Fig. 3B), a result consistent with a self-associating system. Hence, in this case the presence of a single peak in the $g(s^*)$ data shown in Fig. 3A is not indicative of a single sedimenting species. This is because the effects of diffusion in these experiments may conceal heterogeneity, especially in the case where the sedimentation coefficients of all sedimenting species vary only slightly (46). Rather, the ensemble of data suggests a diffuse mixture of species whose sedimentation is dynamic and dependent upon the changing radial concentration distribution over time (47).

To resolve the number of sedimenting species in solutions of N-HisPurβ, direct boundary fitting of sedimentation velocity data were conducted. Despite only observing a single Gaussian peak in the $g(s^*)$ distributions, fitting of radial Δc (subtraction of scan pairs) data to a single sedimenting species model was poor relative to that of a monomer-dimer equilibrium model as judged by an increase in randomness of the residuals and by fitting statistics (Fig. 4). Fitting to the single-species model revealed an apparent 3.97 Svedberg species with an apparent $M_r$ = 66,890.1, which is lower than the expected molecular weight for dimeric N-HisPurβ. This is suggestive of enhanced diffusion due to self-association ($M_r ~ s/D_T$).

Furthermore, fitting to an associating model revealed the sedimentation coefficients and molecular weights for both monomeric and dimeric species. At a loading concentration of 13.4 μM, it was found that the monomeric species sediments with a $s_{20w}$ of 1.79 (1.70 -...
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...of 0.30–0.35 g of H₂O/g of protein are generally used in instances where the degree of hydration was on the order of 10%; however, values of 0.30–0.35 g of H₂O/g of protein were shown that corrections for folding on the degree of hydration are marked different from those found by light-scattering techniques but are in reasonable agreement. The difference is likely due to the fact that DLS measurements are made in a bulk manner on a polydisperse system and, in turn, are weight averages.

Hydrodynamic Shape of Recombinant Purβ—The disparities between the experimentally derived and calculated R₅₀ values as well as the value of f/f₀ for the dimeric species are suggestive that the shape of dimeric N-HisPurβ is markedly different from that of a condensed sphere. Molecular shape calculations predict that N-HisPurβ dimer is elliptical in solution, either prolate or oblate, with axial ratios of either a/b = 7.01 or 8.05 for a prolate or oblate ellipsoid, respectively. These calculations were made using a degree of hydration of 0.43 g of H₂O/g of N-HisPurβ, which represents the predicted value based on amino acid composition of the protein assuming all amino acids are solvent-exposed and hydrated to their identity-specific degrees, disregarding hydration upon folding (48). Given this degree of hydration, the molecular dimensions would be 23.57 × 3.36 nm (2a × 2b) for the prolate prediction and 12.90 × 1.60 nm (2a × 2b) for the oblate ellipsoid. However, studies suggest that the actual degree of hydration of proteins is generally lower than maximal values, presumably due to folding and exclusion of water in the hydrophobic core of proteins. Kuntz (48) showed that corrections for folding on the degree of hydration of proteins were on the order of 10%; however, values of 0.30–0.35 g of H₂O/g of protein are generally used in instances where the actual degree of hydration is not known. Unfortunately, accurate hydrodynamic modeling cannot be accomplished without prior knowledge of either the degree of hydration or the axial ratios of the hydrated molecule (49). If a 10% decrease in hydration of dimeric N-HisPurβ due to folding is assumed, the resulting hydration value of 0.39 g of H₂O/g of protein predicts axial ratios of 7.29 and 8.43 for a prolate and oblate ellipsoid, respectively. Molecular dimensions arising from these values would then be 23.91 × 3.28 nm (2a × 2b) for the prolate case and 12.94 × 1.54 nm (2a × 2b) for the oblate prediction. Hence, the projected dimensions differ only modestly with this assumption-based correction for folding.

Self-association of Mouse Purβ—The disparities between the experimentally derived and calculated R₅₀ values as well as the value of f/f₀ for the dimeric species are suggestive that the shape of dimeric N-HisPurβ is markedly different from that of a condensed sphere. Molecular shape calculations predict that N-HisPurβ dimer is elliptical in solution, either prolate or oblate, with axial ratios of either a/b = 7.01 or 8.05 for a prolate or oblate ellipsoid, respectively. These calculations were made using a degree of hydration of 0.43 g of H₂O/g of N-HisPurβ, which represents the predicted value based on amino acid composition of the protein assuming all amino acids are solvent-exposed and hydrated to their identity-specific degrees, disregarding hydration upon folding (48). Given this degree of hydration, the molecular dimensions would be 23.57 × 3.36 nm (2a × 2b) for the prolate prediction and 12.90 × 1.60 nm (2a × 2b) for the oblate ellipsoid. However, studies suggest that the actual degree of hydration of proteins is generally lower than maximal values, presumably due to folding and exclusion of water in the hydrophobic core of proteins. Kuntz (48) showed that corrections for folding on the degree of hydration of proteins were on the order of 10%; however, values of 0.30–0.35 g of H₂O/g of protein are generally used in instances where the actual degree of hydration is not known. Unfortunately, accurate hydrodynamic modeling cannot be accomplished without prior knowledge of either the degree of hydration or the axial ratios of the hydrated molecule (49). If a 10% decrease in hydration of dimeric N-HisPurβ due to folding is assumed, the resulting hydration value of 0.39 g of H₂O/g of protein predicts axial ratios of 7.29 and 8.43 for a prolate and oblate ellipsoid, respectively. Molecular dimensions arising from these values would then be 23.91 × 3.28 nm (2a × 2b) for the prolate case and 12.94 × 1.54 nm (2a × 2b) for the oblate prediction. Hence, the projected dimensions differ only modestly with this assumption-based correction for folding.

Sedimentation Equilibrium Analysis of Recombinant Purβ—To validate the monomer-dimer self-association model for N-HisPurβ and to confirm the equilibrium constant that characterizes this association, sedimentation equilibrium studies were performed. A 10-fold range of concentrations (from 4 to 40 μM) of size-fractionated N-HisPurβ in buffer E were sedimented at three different rotor speeds until equilibrium was attained (representative scans are shown in Fig. 5). A careful and systematic analysis of experiments in which protein concentration, rotor speeds, and buffer conditions were altered indicated that we were limited in the range of conditions that would result in interpretable data. We were unable to run N-HisPurβ at concentrations lower than 4 μM due to low radial absorbance distributions resulting in values below the signal to noise ratio of the instrument (data not shown). Attempts at lowering the reducing agent concentration to lower base-line absorbance, in an effort to collect low concentration absorbance datasets at 230 nm, resulted in formation of higher order oligomers as assessed by SEC (data not shown). We believe that these were artifactual disulfide oxidation products. Protein concentrations and rotor speeds were chosen such that non-ideal solution conditions were avoided. Furthermore, buffer conditions were selected to ensure N-HisPurβ solubility and stability over the time frame of the experiment.

Our logic in choosing an appropriate set of experimental conditions to perform sedimentation equilibrium experiments was as follows. We evaluated the dependence of the ratio of apparent weight-average molecular weight to the calculated monomeric molecular weight (Mₘ⁰/App/Mₘ⁰/Calc). We describe two possible outcomes. First, a systematic decrease in the Mₘ⁰/App/Mₘ⁰/Calc ratio as a function of loading concentration is suggestive...
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TABLE 1
Sedimentation equilibrium data; parameters from global analysis

| Model                      | Root mean square deviations | Converged parameter |
|----------------------------|----------------------------|---------------------|
| Single, ideal              | 8.81 × 10⁻³                | M_r = 66,489 ± 79   |
| Single, non-ideal          | 9.68 × 10⁻³                | A_p = 4.96 × 10⁻⁷ ± 4.01 × 10⁻⁷ mol/mol/g |
| Monomer-N-mer              | 8.55 × 10⁻³                | N = 2.04 ± 0.03 ln K_p = 13.49 ± 0.14 |
| Monomer-dimer              | 8.53 × 10⁻³                | ln K_p = 13.69 ± 0.10 |
| Monomer-dimer + incompetent monomer | 8.56 × 10⁻³                | ln K_p = 13.37 ± 0.11 α = 0.00 ± 0.00 |
| Monomer-dimer + incompetent dimer | 8.58 × 10⁻³                | ln K_p = 13.70 ± 32.11 α = −0.003 ± 22.79 |

* M is constrained to dimeric molecular weight (70,337.2).
* N is constrained to a value of 2.0 (dimer).

of either hydrodynamic or electrostatic repulsion solution non-ideality. Both situations can be created by high loading concentrations or sedimentation at high rotor speeds and manifest as perturbations of the observed thermodynamic parameters of the system (50). Alternatively, M_w App/M_Calc ratios greater than 1 and/or positive slopes in plots of this kind are indicative of electrostatic attraction. This latter case was seen at loading concentrations below 40 μM and values of M_w App/M_Calc approached 2, a result suggestive of dimerization (supplemental Fig. S1). We found that the radial concentration distributions of N-HisPurβ at sedimentation equilibrium using loading concentrations of 1.40, 0.35, and 0.24 mg/ml (39.8, 9.94, and 3.98 μM, respectively) and rotor speeds of 22,000, 28,000, and 35,000 rpm fit best to an ideal monomer-dimer model, as judged by fitting statistics and inspection of residual plots for systematic deviations (Fig. 5 and Table 1). The global fit of 9 data sets to a reversible monomer-N-mer model returned a value of n = 2.04 ± 0.03 (holding monomer molecular weight = 35,168.6). This result confirmed the monomer-dimer equilibrium model that was suggested by the light scattering and sedimentation velocity data. Attempts at fitting the data to other models, including a single ideal monomer and various association models that incorporated noncompetent monomers or dimers, did not result in improved fits (Table 1). Holding stoichiometry constant at n = 2, global fitting of the data yielded a dissociation constant, K_d = 1.13 μM ± 0.27 μM (Table 1; ± 1 S.D.), which is reasonably consistent with direct sedimentation velocity boundary fitting analyses.

DISCUSSION

In this report we utilized quantitative biophysical approaches to study the quaternary structure of recombinant mouse Purβ in the absence of nucleic acid. Incorporation of a SEC step during the purification process coupled with hydrodynamic and thermodynamic analyses allowed us to rigorously and conclusively characterize the solution state of Purβ as a reversible monomer-dimer equilibrium. The value of K_d reported here should be interpreted as an upper limit, since all equilibrium measurements were made at concentrations greater than the apparent value of ~1 μM (Fig. 6). This was a technical necessity as the instability of N-HisPurβ in the absence of reducing agent together with its low UV absorbance signature prevented the acquisition of interpretable radial absorbance data with dilute solutions of protein. Hence, it is likely that equilibrium measurements of Purβ self-association at concentrations lower than those utilized here will have to be made with protein labeled with an extrinsic chromophore (51) or by use of a sensitive orthogonal technique such as fluorescence polarization spectroscopy (52). In a related matter, the limited solubility of the protein below a 200 mM threshold of NaCl hindered the hydrodynamic assessment of ionic contributions to N-HisPurβ self-association. The consequences of increased ionic strength and/or alternative monovalent salts on self-association energetics have not yet been rigorously investigated.

Hydrodynamic radius determinations by dynamic light scattering or by extrapolation from sedimentation data were in sound agreement. Molecular shape calculations were consistent with an asymmetric shape of dimeric N-HisPurβ in solution. This finding is not surprising based on the fact that Purβ is composed of 22.2% glycine (13), which likely contributes to a lack of secondary structural elements and a somewhat disordered tertiary structure and may explain difficulties in obtaining higher resolution structural information. A further interesting finding is that the f/f_o ratio determined for the monomeric species is greater than that of the dimer, which suggests that dimerization results in partial condensation of the overall structure. Implications of this finding are discussed below.

We also addressed the concern that the N-terminal hexahistidine tag present on the recombinant protein might adversely affect the functional activity of Purβ by comparing the ssDNA binding activity of native and recombinant N-HisPurβ using a...
quantitative enzyme-linked immunosorbent assay approach (21). The ssDNA binding activity of N-HisPurβ was indistinguishable compared with Purβ derived from either mouse embryo fibroblasts or vascular smooth muscle cells (data not shown). Also, nonspecific metal ion-mediated dimerization of hexahistidine can be eliminated as a complicating factor since all measurements were made in the presence of 0.5 mM EDTA.

Our interest in Pur proteins stems from their putative involvement in repressing the transcription and translation of genes that mark the phenotypic status of myofibroblasts, vascular smooth muscle cells, and cardiomyocytes (14, 15, 21, 23, 29). Evidence for formation of transient ssDNA structures within the asymmetric purine/pyrimidine tract of the 5′-3′ SmA promoter (33) has also fueled investigation into the mechanism of DNA binding by Pur proteins. It has been suggested that Pur proteins bind PUR elements as either hetero- or homodimers (22), although the stoichiometry, mechanism, and thermodynamics of nucleoprotein assembly have not yet been determined by rigorous physical investigation. In the case of Purα, self-association has been reported to be mediated by an RNA molecule of unknown nature (34). This was an intriguing finding, as it suggested that RNA may influence the ability of Purα to stably self-associate and to bind to ssDNA. Curiously, despite sharing 70% amino acid sequence identity, we have shown in this study that Purβ dimerizes in the absence of any nucleic acid. It is possible that this disparity is due to a distinct functional difference between Purα and Purβ as implied by previous gain-of-function studies (21, 29) or could be reflective of different experimental approaches (e.g. pulldown assay versus sedimentation equilibrium analysis). Moreover, the apparent binding affinities of Purα and Purβ for different PUR elements are reported to be on the order of 1 nM (21, 29, 32), whereas the affinity of Purβ self-association is 3 orders of magnitude weaker. As such, Purβ at concentrations below 100 nM is predicted to be largely monomeric as depicted in the molecular species plot in Fig. 6. This suggests that other factors (such as an RNA ligand in the specific case of Purα) may be required to help facilitate dimerization in a cellular milieu if the concentration of protein is limiting. On the other hand, it remains to be resolved as to what extent Purα and Purβ share similar intrinsic homodimerization ability in the absence of nucleic acid and whether or not heterodimeric complexes associate with enhanced or reduced affinity relative to their homodimeric counterparts.

In conclusion, we report that recombinant Purβ participates in a monomer-dimer equilibrium governed by an apparent upper limit dissociation constant of ∼1 μM. The ability to dimerize in a reversible fashion may represent an important regulatory mechanism, allowing mass-action governed self-association to play either a positive or negative role in nucleic acid binding. As indicated by frictional coefficient ratios, dimerization may result in structural reorganization of N-HisPurβ that may permit nucleic acid recognition and binding. Self-association of transcription factors as a prerequisite to DNA binding is not unprecedented. For example, the STAT proteins require phosphorylation-dependent dimerization before nuclear localization and binding to cytokine responsive gene promoters (53). Similarly, intracellular estrogen receptor DNA binding activity is apparently dependent upon ligand-mediated dimerization (54–56). However, in the case of Purβ, dimerization does not appear to require post-translational modification or ligand binding per se as self-association is governed by protein concentration in vitro. This fact does not rule out the prospect that post-translational modifications may alter the affinity of homodimerization in vivo. Although the absolute intracellular concentration is not known, it has been reported that levels of Purβ increase dramatically in cardiovascular cell types undergoing phenotypic changes (15, 35). This enforces speculation that repressive effects of Purβ may depend upon its expression and accumulation within the nucleus to levels that drive self-association and permit ssDNA binding. Future studies will focus on determining the affinity and stoichiometry of relevant Pur nucleoprotein complexes with the use of quantitative biochemical approaches such as those described herein.

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