Quarternary structure amplification of protein folding differences observed in 'native' platelet factor-4

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Abstract Platelet factor 4 (PF4), a protein of 70 residues, exists in solution as a distribution of monomer, dimer and tetramer (M-D-T) states in slow exchange on a 600 MHz ^{1}H-NMR chemical shift time scale. Well-resolved Y60 ring proton resonances in each aggregate state allow derivation of M-D-T populations. Under the same set of solution conditions, M-D-T aggregate state distributions vary from prep to prep or with repeated freeze-drying or raising/lowering the solution pH. These variations are not the result of chemical modifications and reflect differences in the strength of subunit associations and therefore folding. Variations are greatest at pH values at or below the pK_as of carboxylate groups, supporting the idea that electrostatic interactions modulate PF4 subunit interactions. Treating these distributions as true equilibria results in free energy differences of about 0.5 kcal/mol subunit. Quarternary structure amplifies free energy differences among various folded states of monomer PF4.

Key words: Protein folding; NMR; Platelet factor 4; Subunit association

1. Introduction

Native human platelet factor 4 (PF4) (7,800 Da in the monomer state) forms an anti-parallel β-sheet structural scaffold onto which is folded a C-terminal α-helix and an aperiodic N-terminal domain [1,2]. X-ray structure analysis of homotetrameric human [1] and bovine [3] PF4 shows that the crystal arrangement of the four monomer subunits (identified as A,B,C and D) is asymmetric with most of the asymmetry found at AC and AD-type dimer interfaces. Similar observations have been made for tetramer human PF4 in solution [2]. AD-type dimers form the weakest interactions. Two Glu-Lys salt bridges and hydrophobic side-chain interactions stabilize AC-type dimers, while anti-parallel β-sheet hydrogen bonds and hydrophobic interactions stabilize AB-type dimer interactions. Overall, electrostatic interactions are crucial to subunit association, i.e. quarternary structure folding [1,4,5].

Protein folding is primarily dictated by non-covalent, relatively weak intermolecular forces [6] which, at room temperature, should allow for relatively rapid conformational reshuffling [7]. Studying partially folded states and multiple conformations has generally required the use of indirect methods for their investigation. The existence of multiple conformation minima with small energy differences (e.g. 0.4 kcal/mol), for example, was first recognized in myoglobin by using low temperature (less than about 200 K) X-ray diffraction analyses in order to 'freeze out' normally rapidly fluctuating structural states [8,9]. By using NMR and proton/deuteron exchange methods, several groups [10,11,12] have assayed the 'structural' state of individual NH groups at various points along various protein folding pathways. Fersht et al. [12,13] have used protein engineering and refolding kinetics to deduce the presence of folding transients and intermediate states. Methyl group-sized hydrophobic cavities have been engineered in barnase [12,13] and T4-lysozyme [14] to assess energetic differences among folded states by using X-ray crystallography and calorimetry.

While these approaches have addressed important aspects of protein folding at the secondary and tertiary structure levels, little information is available concerning quarternary structure folding. Under certain solution conditions (i.e. mg protein/ml, pH 2–5, low ionic strength and temperatures less than 50°C), ^{1}H-NMR spectra of PF4 show multiple resonances due to the presence of M-D-T aggregate states which exchange slowly on the chemical shift time scale [4,5]. The simplicity of the aromatic region (H23, H35 and Y60) allows resolution of Y60 2,6 and 3,5 proton resonances in each aggregate state from which M-D-T populations can be derived. During the course of numerous protein isolations of PF4, it became apparent that these M-D-T distributions vary somewhat from prep to prep (either with PF4 isolated from platelets or with recombinant protein) and even within the same prep, distributions vary from lyophilization to lyophilization or by repeatedly raising and lowering the pH. Here, small energetic differences among PF4 folded states can be indirectly differentiated due to amplification by subunit association.

2. Materials and methods

2.1. Isolation of native PF4

For PF4, outdated human platelets were obtained from the Red Cross and centrifuged at 10,000 × *g* for one hour to obtain platelet poor plasma. This preparation was applied to a heparin-agarose (Sigma) column (bed volume 50 ml); the column was washed with 0.2 M, 0.5 M, 1 M, and 1.5 m NaCl. The fraction eluting at 1.5 M NaCl which yielded most of the PF4 [15] was then desalted by dialysis (0.2% trifluoroacetic acid). The resulting solution was concentrated by lyophilization and purified further by HPLC as discussed by Mayo and Chen [4,5]. From about 50 units of outdated platelets, 20 mg of PF4 generally resulted.

2.2. Protein concentration

Protein concentration was determined by the method of Lowry et al. [16] and results were calculated from a standard dilution curve of human serum albumin. An alternative method used to determine PF4 concentration was that of Waddell [17].

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Abbreviations: PF4, platelet factor 4; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; rf, radio frequency; FID, free induction decay; CD, circular dichroism; HPLC, high performance liquid chromatography; M, monomer; D, dimer; T, tetramer.

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2.3. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H-NMR spectra were recorded in the Fourier mode on a Bruker AMX-600 spectrometer (600 MHz for protons). The solvent deuterium signal was used as a field-frequency lock. All chemical shifts are quoted in parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentane sulfonate (DSS).

5 mg PF4 was dissolved in 0.6 ml deuterium oxide (no added buffer) with 2 mM NaCl and the pH was adjusted to pH 2.5 in the NMR tube. ^{1}H NMR (600 MHz) spectra were accumulated at 30°C every 0.1 to 0.3 pH units up to pH 5. The pH was then lowered to pH 2.5 and the cycle repeated up to five times. The p^{2}H was adjusted by adding ml increments (minimal volume) of NaO^{2}H or ^{2}HCl to the sample. All measurements were done at the p^{2}H value indicated in the text read directly from the pH meter and not adjusted for isotope effects.

2.4. Gaussian/Lorentzian line-shape analysis

Resonance area integrals were derived by Gaussian/Lorentzian line fitting of NMR spectra directly on the spectrometer or offline on a Bruker Aspect-1 workstation with standard Gaussian/Lorentzian functions. A baseline was established by comparing fits with the noise level where no resonances were found on both sides of the aromatic resonance region. Baselines were generally flat. Derived areas from these fits varied by no more than about 10%.

3. Results and discussion

Fig. 1 shows three spectra of PF4 accumulated under the same set of solution conditions (pH 4, 30°C and 2 mM NaCl), which typify normally observed M-D-T distributional variations. Here, data have been acquired on one PF4 sample where the solution pH was systematically raised from pH 2.5 to pH 5 and then lowered back to pH 2.5 to restart the cycle. Data for these cyclic pH variations are shown since work could be systematically done on one sample with essentially the same concentration. Similar M-D-T distributional variations have been noted from PF4 prep to prep, or they can be generated by repeated freeze-drying/re-solvation. Fig. 2 plots the natural logarithm of the ratio of [D]/[M] or [T]/[D] versus the solution pH for five pH cycles. Only ratios for pH values between pH 3.4 and pH 4.6 are shown. Above about pH 4.6, the equilibrium shifts to mostly tetramer and significant monomer and dimer populations are not observed. Below about pH 3.4, dimer populations are highly attenuated and only M-T populations can be accurately derived (data not shown). M-D-T distributions vary most at lower pH values consistent with the observation that electrostatic interactions modulate subunit interactions [1,4,5].

From pH (or freeze-dry) cycle to cycle, there is no systematic increase or decrease of any one aggregate state population over another as one might expect if some type of chemical modification or increase in some solute concentration, e.g. salt from pH adjustments, were occurring. Rather there seems to be a random M-D-T distribution change occurring within a narrow range as indicated in Figs. 1 and 2. Over time (several days at room temperature), distributions do not change, and only shifts in pH (or freeze-drying/resolvation) affect the distribution. Raising the temperature up to 60°C also has no apparent effect. N-terminal sequencing, gel electrophoresis, HPLC, and mass

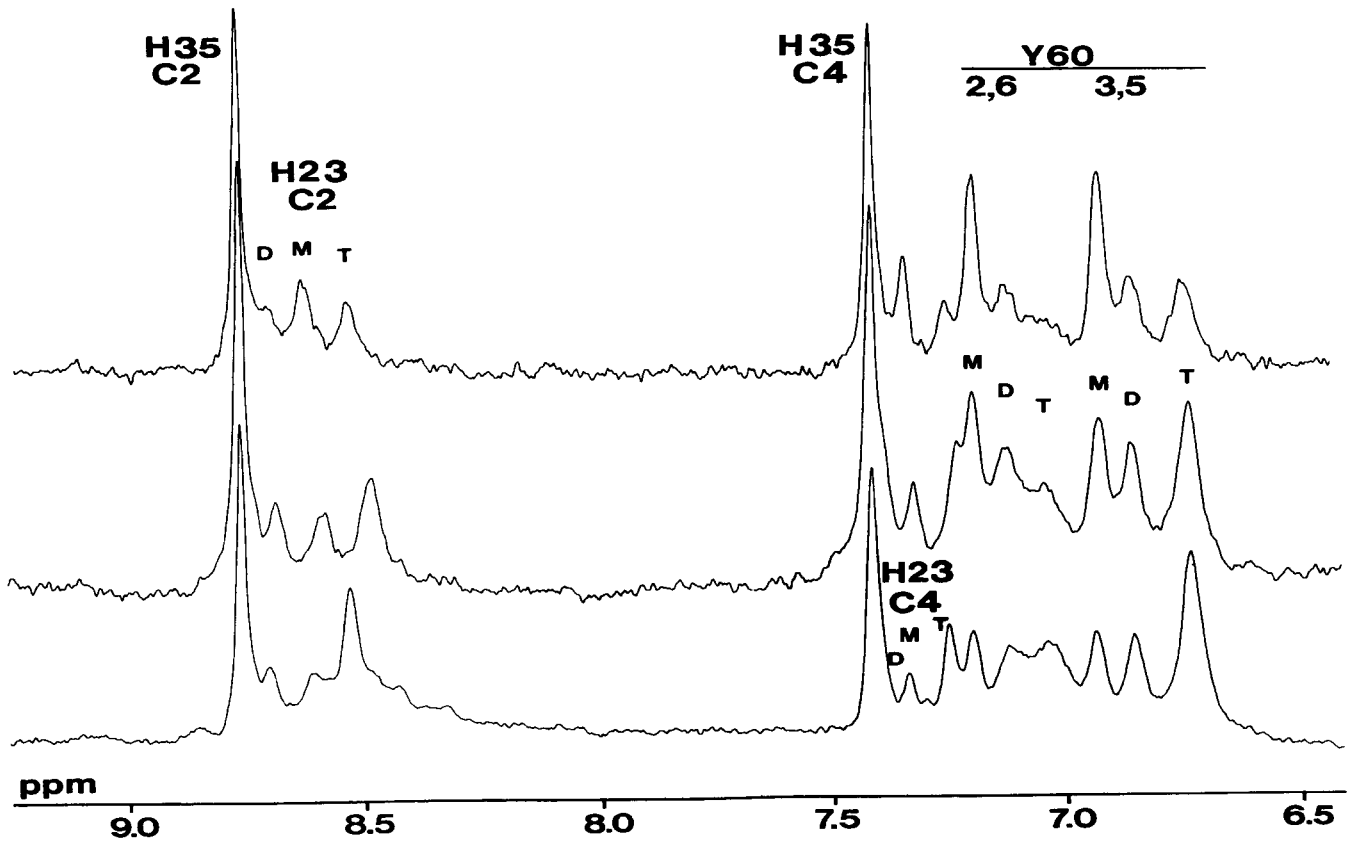

Fig. 1. 600 MHz ^{1}H-NMR spectra from three raising/lowering pH cycles are shown. Spectra were accumulated at 303 K on a Bruker AMX-600 NMR spectrometer under conditions described by Mayo and Chen [4], as a function of pH from pH 2.5 to pH 5. NMR spectra shown, however, were accumulated at pH 4 so that significant populations from all aggregate states could be observed. The resonance label prefixes, M, D and T, stand for monomer, dimer and tetramer states, respectively. Monomer concentrations range from a low of 0.12 mM to a high of 0.24 mM, while tetramer concentrations range from a low of 0.03 mM to a high of 0.06 mM. For these NMR experiments, the sensitivity limit is about 0.005 mM.

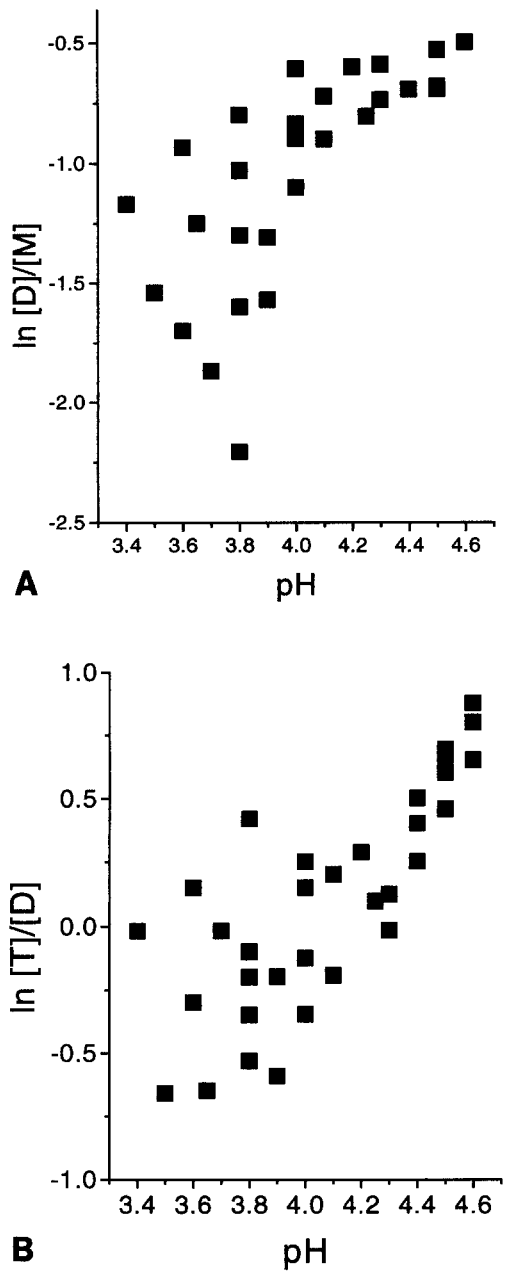

Fig. 2. Plots are shown for PF4 for the natural logarithms of the ratio of [D]/[M] (panel A) and [T]/[D] (panel B) versus pH. These were derived from Lorentzian/Gaussian fits to NMR spectra like those shown in Fig. 1 and as discussed by Mayo and Chen [4,5]. By this method, PF4 aggregate state populations can be measured at better than 10% error. A 10% change in M-D-T populations, for example, yields a fractional change in the natural logarithm of [D]/[M] or [T]/[D] as presented in this figure.

spectrometry indicate chemical identity of all PF4 samples. Furthermore, the oxidation state of the disulfides as measured by the method of Toyo'oka and Imai [18], remains unchanged, i.e. the two disulfide bonds remain intact. In addition, the disulfide-bond reduced state of PF4 has been studied previously and observed changes are not consistent with those on the reduced state [19].

Variations in M-D-T distributions, therefore, reflect differences in the strength of subunit interactions (quarternary structure) and ultimately differences in monomer subunit folding. Although M-D-T distributions technically might be viewed as 'irreversible' and therefore, not representing true equilibria as previously presented [4,5], they are 'reversible' within a specific range. In particular, at pH values above about pH 4.2, distributions vary little and appear to approach a limiting value. Treating these distributions, to a first approximation, as real equilibria yields an overall change in free energy of subunit association, i.e. $\Delta\Delta G_{D(or\ T)}$ (calculated from $\Delta G_{D(or\ T)} = -RT \ln K_{D(or\ T)}$ as $(\Delta G^{i}_{D(or\ T)} - \Delta G^{k}_{D(or\ T)})$] at pH 3.8 of about 0.5 kcal/mol subunit. This value effectively is the maximal free energy difference between different subunit folds. For the folding of barnase [12,13], free energy differences of 1–2 kcal/mol have been correlated with specific structural perturbations which destabilize folded states by creating methyl group-sized cavities. In T4-lysozyme [14], various hydrophobic core 'cavity creating' mutants affected protein stability at pH 3 by 2 to 5 kcal/mol. Free energy changes in PF4 folding observed here are on this order of magnitude. In this respect, a slight, but significant, conformational change in PF4 would account for the approximated free energy change.

Without knowing individual 'sub-state' structures, correlation of these free energy differences with specific conformational perturbations in PF4 is not possible. CD and NMR NOE results, however, indicate that global folding is not affected, consistent with the magnitude of free energy change noted above. Furthermore, the overall trends on varying solution conditions hold as originally observed [4,5] supporting the idea that subunit interactions also are generally conserved. A shallower slope of ln [D]/[M] or ln [T]/[D] versus pH indicates, however, relatively fewer and/or weaker electrostatic interactions [20]. This suggests that although conformational changes are apparently slight, they do perturb spatial orientations of charged side-chains. In line with this is the observation that H23 ring 2H resonances are shifted when in different states (Fig. 1). This indicates slight H23 side-chain pK_a shifts consistent with a conformational change at H23. The H23 pK_a also lies between pH 4 and pH 5 [4].

Initially, the idea that folding differences could be responsible for these M-D-T distribution variations was difficult to accept since most proteins studied to date fold on the order of seconds [6] and PF4 subunit exchange lifetimes (about 0.1 s) [5] should give sufficient time for simple conformational reshuffling [7]. On the other hand, the time for PF4 folding is unknown and some proteins are known to fold on the order of minutes or hours [21]. In addition, some physical properties of proteins suggest that they are glass-like substances [9,22,23] where very long relaxation times between/among states are common. PF4 may be showing glass-like properties at room temperature. One possible cause for these folding variations in PF4 could be *cis-trans* proline isomerization which is generally known to be slow on the folding time scale [6,24]. However, all four prolines in PF4 are observed to be in the *trans* isomer state in both crystal [1] and solution [2] structures. Since M-D-T distributions do not change on merely sitting or raising the temperature, energy barrier heights among conformational minima must be relatively high and/or conformational reshuffling must involve many concerted steps or highly correlated fluctuations. M-D-T subunit exchange may not give sufficient time for reshuffling and may effectively trap [25–29] se-

lected conformations. These traps can only be breached by freeze-drying/re-solvation (denaturation/renaturation) or by raising/ lowering the solution pH.

It is easier to accept this when distributions change as a result of freeze-drying/ re-solvation, however, it is not immediately apparent why changing the pH would effect such change. PF4, however, is an unusual protein in that it is highly hydrophobic within its interior sequence and highly charged at its termini with positive charges clustering at the C-terminus and negative charges clustering at the N-terminus. Moreover, native PF4 is a homotetramer whose subunits are asymmetrically associated [1,2] and stabilized by electrostatic interactions within a low dielectric inter-subunit interior [4,5]. Since observed differences in M-D-T distributions are most apparent at pHs below the pK_a values of carboxylates, this supports the significance of electrostatic interactions in modulating PF4 subunit associations [1,4,5]. During pH cycles, raising the pH above about 4.5 effects the greatest change in M-D-T distributions. As subunits pass through the carboxylate pK_a range, the ensemble of charged/ uncharged carboxylate groups associates in various combinations and permutations of dimeric/tetrameric species. We propose that quarternary structural interactions in the native asymmetric tetramer vary enough to induce slight tertiary conformational changes which become trapped by the equilibrium kinetics of the association process. Accidently selected PF4 folds are observed by NMR due primarily to the effect of pH and quarternary structure amplification of monomer fold free energy differences. Amplification results from the squared term which appears when relating tertiary folding and subunit association equilibria. Such small energetic differences would be difficult, if not impossible, to observe by most analytic techniques. Quarternary structure amplification of monomer fold free energy differences may be more common place as more quarternary structural proteins are investigated.

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