Conformational stabilities of the rat α- and β-parvalbumins

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Abstract It is widely believed that β-parvalbumin (PV) isoforms are intrinsically less stable than α-parvalbumins, due to greater electrostatic repulsion and an abbreviated C-terminal helix. However, when examined by differential scanning calorimetry, the apo-form of the rat β-PV (i.e. oncomodulin) actually displays greater thermal stability than the α-PV. Whereas the melting temperature of the α isoform is 45.8°C at physiological pH and ionic strength, the T_m for the β isoform is more than 7° higher (53.6°C). This result suggests that factors besides net charge and C-terminal helix length strongly influence parvalbumin conformational stability. Extension of the F helix in the β-PV, by insertion of Ser-109, has a modest stabilizing effect, raising the T_m by 1.1°. Truncation of the α-PV F helix, by removal of Glu-108, has a more profound impact, lowering the T_m by 4.0°.

Key words: Calcium-binding protein; Parvalbumin; Protein stability; Calorimetry

1. Introduction

The parvalbumin (PV) family of EF-hand proteins [1–4] includes α and β sub-lineages [5,6]. The α isoforms are less acidic (p$I \geq 5$) than their β counterparts and have an additional amino acid residue in the C-terminal helix. The mammalian genome encodes one isoform from each lineage. The α-PV has a relatively broad tissue distribution – very abundant in fast-twitch skeletal muscle and present at lower levels in select neurons, kidney, adipose tissue, testis, and ovary [4]. The protein exhibits very high affinity for Ca^{2+} and moderate affinity for Mg^{2+} [7–9], properties consistent with a Ca^{2+}-buffering role.

By contrast, the mammalian β-PV (also known as oncomodulin) has a very narrow tissue distribution. Postnatal expression of the β isoform is apparently restricted to the outer hair cells in the organ of Corti, the mammalian auditory organ [10–12]. Although expression has also been detected in rat placenta and pre-implantation embryo [13,14], its physiological relevance in these settings is questionable [15]. The function of oncomodulin is unknown. Relative to the α isoform, the β-PV displays highly attenuated affinity for Ca^{2+} and Mg^{2+} [16,17], fueling speculation that it functions as a tissue-specific Ca^{2+}-dependent regulatory protein. However, no target protein has been identified to date, and it is equally possible that oncomodulin serves as a specialized Ca^{2+} buffer protein.

Long-standing favorites for physical characterization, parvalbumins have been examined by virtually every physical and spectroscopic technique. From these myriad studies, a consensus opinion has emerged that the β-PVs are intrinsically less stable than their α counterparts (e.g. [18–20]). Their reduced stability has been attributed to greater electrostatic destabilization and the shorter C-terminal helix. In the interest of evaluating the relative importance of these two factors, we plan to examine the thermal stabilities of select site-specific variants of the rat α- and β-parvalbumins. While examining the stabilities of the wild-type proteins, we unexpectedly observed that the apo-form of the β isoform in fact displays greater thermal stability than the apo-α-PV. Our analysis is presented below.

2. Materials and methods

2.1. Protein expression and mutagenesis

The rat α- and β-parvalbumin isoforms were expressed in *Escherichia coli* DH5α, using the pLD2 expression vector, a derivative of pBluescript in which transcription is driven by the *lac* promoter. Alterations in the coding sequences were introduced by oligonucleotide-directed mutagenesis, employing a double-stranded template [21]. The proteins were typically purified from 1-l stationary-phase cultures in LB broth containing ampicillin (100 μg/ml). The isolation of the β-PV has been described previously [16].

2.2. Purification of the recombinant rat α-PV

Recombinant bacteria were lysed in 20 mM HEPES-NaOH, pH 7.4, by treatment with hen egg lysozyme (5 mg/g cell paste) for 15 min at 37°C, followed by passage through a French pressure cell. The resulting lysate was heated to 80°C, maintained at 80°C for 5 min, cooled to 4°C in an ice-water bath, and centrifuged (27 000×*g*, 20 min, 4°C). After dialysis for 36 h at 4°C against 1 mM EDTA, pH 7.4, the supernatant liquid was loaded onto DEAE-Sepharose (30 ml bed volume) that had been previously adjusted to pH 7.4 and rinsed with several bed volumes of water.

After removing unbound proteins with one bed volume of 1 mM EDTA, pH 7.4, the column was eluted with a 0–50 mM $CaCl_2$ gradient (240 ml total volume) in 5 mM HEPES, pH 7.4. Since the protein has a characteristic UV signature (8 phenylalanines, no tryptophan or tyrosine) [22,23], fractions containing the α-PV could be identified by UV spectroscopy. Remaining contaminants were removed by gel-filtration on Sephadex G-75 at room temperature, in 0.15 M NaCl, 0.025 M HEPES, pH 7.4. A 1-l culture typically yields 40–60 mg of pure protein.

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) data were collected with a Nano-DSC (Calorimetry Sciences Corp., Provo, UT). All scans were obtained with a heating rate of 1°C/min, after verifying that the width and shape of the transitions were independent of scan temperature at or below this value. Samples (2–4 mg/ml) were dialyzed to equilibrium against the reference buffer (0.20 M NaCl, 10 mM EDTA, pH 7.4). After obtaining a baseline scan with buffer alone in both sample and reference cells, the cells were refilled – with the protein solution and buffer, respectively – and rescanned.

The thermal transitions of both rat parvalbumins are highly reversible under the conditions employed for this study. Heating, cooling, and re-heating scans are displayed for the β isoform in Fig. 1. The area under the second denaturation endotherm is 96% of the original. However, if the upper scan limit is increased above 80°C, diminished reversibility is observed, possibly due to chemical modification (e.g. oxidation, hydrolysis) of specific residues in the proteins.

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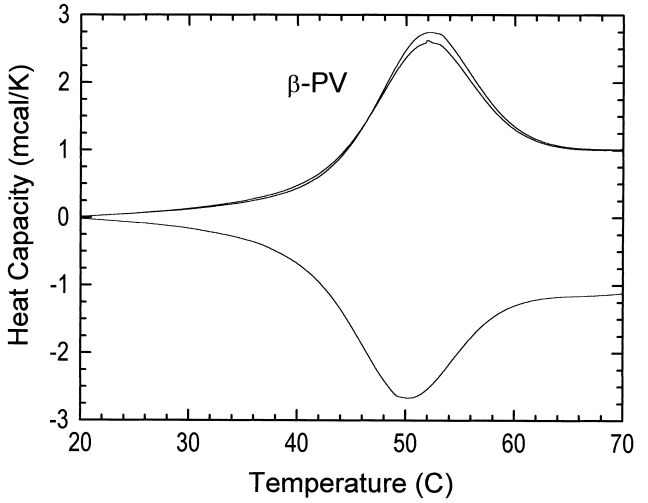

Fig. 1. Successive DSC traces are shown for a sample of rat β-PV. Notice the prominent exotherm in the intervening cooling scan.

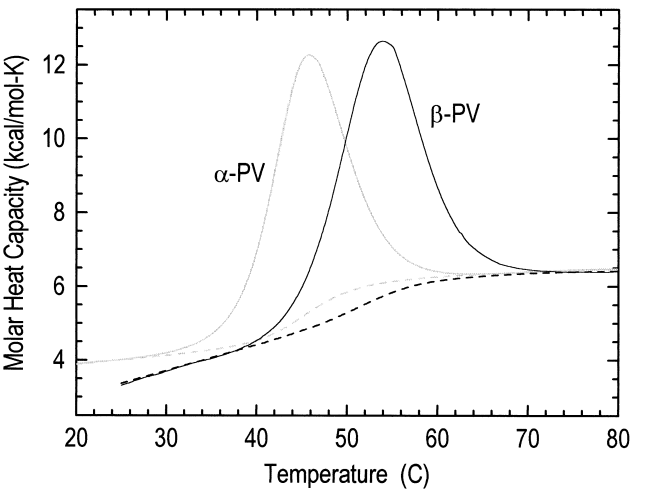

Fig. 2. Thermal denaturation of rat α-PV (solid gray line) and rat β-PV (solid black line). DSC was carried out as described in Section 2. The corresponding dashed lines indicate the calculated baselines in the region of the thermal transition.

Data were analyzed with CpCalc, v.2.0 (Applied Thermodynamics Corp.). Upon importation into CpCalc, raw power data are automatically converted to heat capacity. Following subtraction of the baseline scan, the sample scan was converted to molar heat capacity. Baselines were fitted to the pre- and post-transition regions, using a linear function for the pre-transition region and a quadratic expression for the post-transition region. CpCalc was then used to calculate the T_m and the enthalpy and entropy of denaturation at that temperature.

3. Results

3.1. Comparison of rat α- and β-PV thermal stability

Representative scanning calorimetry data for the rat α- and β-parvalbumins – obtained in 0.20 M NaCl, 0.01 M EDTA, pH 7.4 – are presented in Fig. 2. Under these conditions, the α-PV denatures at 45.8°C, with an accompanying enthalpy change of 70 ± 2 kcal/mol. Under the same conditions, the β-PV denatures at 53.6°C, with an enthalpy change of 75 ± 3 kcal/mol. Thermodynamic parameters for the two proteins are listed in Table 1.

The apparent, or van't Hoff, enthalpy (ΔH_{vh}) for a process is obtained from the temperature dependence of the equilibrium constant. If the reaction under consideration is a two-state process, the ratio of van't Hoff and calorimetric enthalpies ($\Delta H_{vH}/\Delta H_{cal}$) should be unity (e.g. [24]). ΔH_{vh} for protein denaturation can be estimated from DSC data by means of the following equation [25]:

$$\Delta H_{vH} = 4RT_m^2 \frac{C_{p,ex}(T_m)}{\Delta H_{cal}} \quad (1)$$

where $C_{p,ex}(T_m)$ is the excess molar heat capacity at the melting temperature (i.e. the measured heat capacity at T_m minus the baseline heat capacity), ΔH_{cal} is the calorimetric enthalpy, and R is the gas constant. Eq. 1 yields a van't Hoff enthalpy of 71 kcal/mol for the α-PV. The solid line in Fig. 3A shows the agreement between the observed excess heat capacity and the values calculated with this estimate of ΔH_{vH}. Application of Eq. 1 to the β-PV data yields an apparent enthalpy of denaturation equal to 74 kcal/mol. Fig. 3B similarly shows the agreement between the observed and calculated excess heat capacity values for the β isoform. The near equality of the van't Hoff and calorimetric enthalpies suggests that both proteins denature cooperatively without significant population of intermediate states.We can predict the conformational free energies (ΔG_{conf}) of the rat α and β isoforms – i.e. the energies required for denaturation – by means of the following equation (e.g. [26]):

$$\Delta G_{conf} = \Delta H_d(T_m) - T\Delta S_d(T_m) + \Delta C_p[(T - T_m) - T\ln(T/T_m)] \quad (2)$$

where T is the temperature of interest, T_m is the melting temperature, $\Delta H_d(T_m)$ and $\Delta S_d(T_m)$ are the enthalpy and entropy, respectively, for denaturation at the melting temperature, and ΔC_p is the change in the molar heat capacity of the protein upon denaturation. The magnitude of ΔC_p is largely determined by the increase in solvent-accessible apolar surface area that accompanies protein unfolding (e.g. [27]). A priori, then, the rat α and β isoforms should have similar ΔC_p values, given the similarity of their tertiary structures. Utilizing the value of 1.33 kcal mol^{-1} K^{-1} determined by Filimonov et al. [28] for the carp β-PV (pI 4.25), we predict conformational free energies at 25°C of 3.5 kcal/mol and 4.7 kcal/mol for the α- and β-PVs, respectively.

Table 1
Summary of DSC data on wild-type and variant rat parvalbumins

Protein	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$\Delta H_{vH}/\Delta H_{cal}$
rat α-PV	45.8 ± 0.5	70 ± 2	71	1.01
α-PV Δ108E	41.8 ± 0.5	62 ± 2	63	1.02
rat β-PV	53.6 ± 0.5	75 ± 3	74	0.99
β-PV 109S	54.7 ± 0.5	73 ± 3	71	0.97

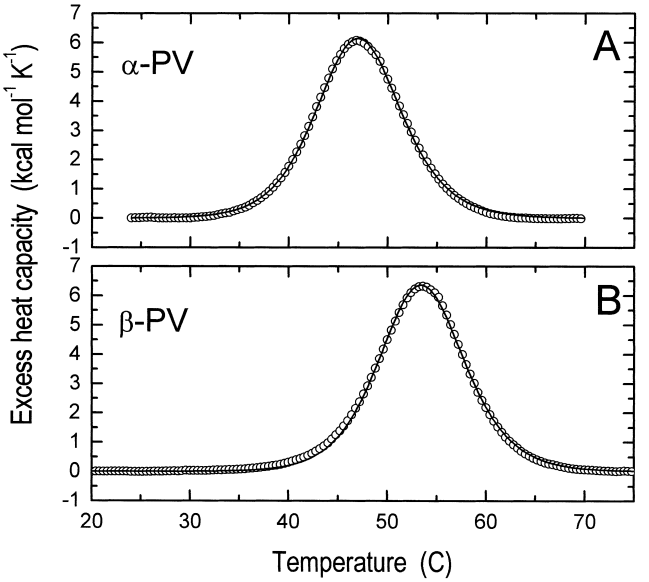

Fig. 3. Two-state denaturation of the rat α- and β-PVs. Observed (◯) and calculated (solid line) values of the excess heat capacity are plotted versus temperature for the α- and β-PV isoforms in panels A and B, respectively. See text for details.

3.2. Influence of C-terminal helix length on PV stability

In the α-PVs, the F helix extends eight residues beyond the $-z$ ligand in the EF binding site. In members of the β sublineage, however, the F helix extends just seven residues beyond the $-z$ ligand. It has been suggested that the additional residue in the α-PV C-terminal helix contributes to the protein's stability – allowing stabilizing tertiary interactions with residues in the B/C region of the molecule [29–31]. We are investigating this hypothesis by site-directed mutagenesis, examining the consequences of extending the F helix in the rat β isoform and of truncating the helix in the α isoform.

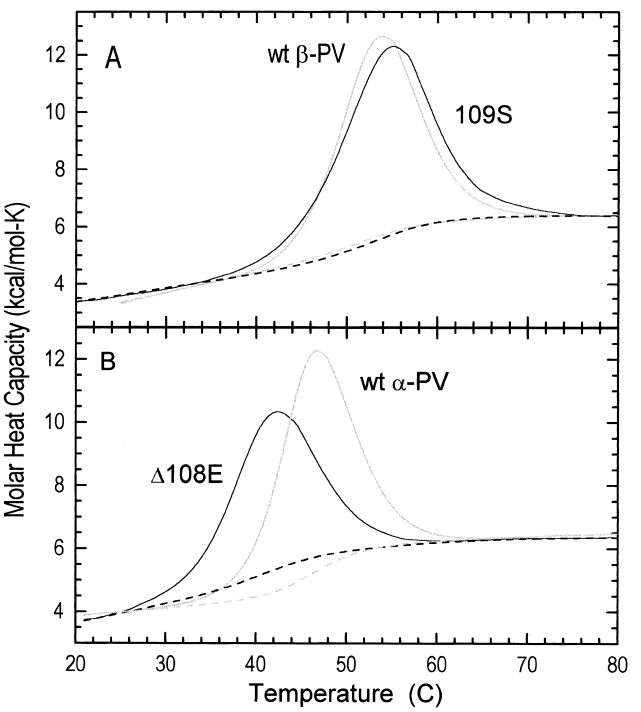

Fig. 4. Influence of C-terminal helix length on α- and β-PV stability. A: Representative DSC traces are presented for the wild-type β isoform (dashed gray line) and for the S109 variant (solid black line). B: DSC data are shown for the wild-type α-PV (solid gray line) and for the Δ108E variant (solid black line). The corresponding dashed lines in panels A and B indicate the calculated baselines in the region of the thermal transition.

DSC data for the 109S variant of the rat β-PV are displayed in Fig. 4A (solid black line), together with corresponding data for the wild-type protein (solid gray line). This mutation – the addition of a serine residue to the C-terminus – increases the melting temperature by 1.1°C, to 54.7°C. Assuming that ΔC_p

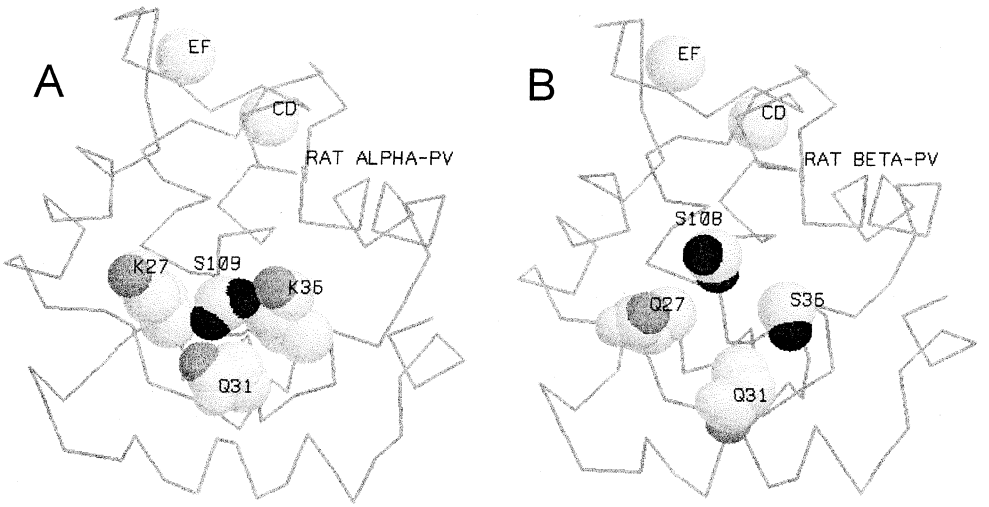

Fig. 5. α-PV-specific tertiary interactions. A: The C-terminal carboxylate of Ser-109 can form salt-bridges or H-bonds with the side chains of Lys-27, Gln-31, and Lys-36 in the α isoform. The carboxylate oxygen atoms are shown in black; the side chain nitrogen atoms of Lys-27, Gln-31, and Lys-36 are depicted in gray. B: Truncation of the F helix at residue 108 prevents the corresponding tertiary interactions in the β-PV. Notice, however, that the carboxylate of Ser-108 could potentially H-bond with the side chain amide proton of Gln-27. The carboxylate oxygen atoms of S108 and the hydroxyl oxygen of S36 are shown in black; the side chain nitrogen atoms of Gln-27 and Gln-31 are depicted in gray. This figure was produced with RasMol [34], using the α-PV X-ray coordinates reported by McPhalen et al. [31] and the β-PV coordinates reported by Ahmed et al. [35].

is unchanged by the mutation, the resulting increase in stability ($\Delta\Delta G_{conf}$) is given by the following equation [26]:

$$\Delta\Delta G_{conf} = \Delta T(\Delta H_d/T_m) \quad (3)$$

where ΔT is the change in melting temperature and ΔH_d is the enthalpy of denaturation of the wild-type protein. Thus, the 1.1° in T_m increase translates into a modest 0.25 kcal/mol increase in stability.

Conversely, what is the effect of truncating the α-PV F helix? DSC data for the Δ108E variant of the rat α-PV are presented in 4B. In Δ108E, the F helix has been shortened by one residue by eliminating Glu-108. This mutation significantly lowers conformational stability, dropping the T_m by 4.0°, to 41.8°C. Assuming again that ΔC_p is unaltered by the mutation, application of Eq. 3 implies that the conformational free energy has been reduced by 0.88 kcal/mol.

4. Discussion

The issue of parvalbumin stability has been addressed previously. Privalov and co-workers reported scanning calorimetry data for one of the carp β isoforms [28], and Sykes and colleagues presented a detailed spectroscopic analysis of rat α-PV stability [32]. However, this study represents the first side-by-side comparison of α- and β-PV stability employing differential scanning calorimetry. Our central observation – i.e. that the rat β-PV isoform denatures with a higher T_m than the α isoform under identical solution conditions – is significant, because it contradicts prevailing opinion on the subject.

A priori, the apo-form of the rat α-PV – with a predicted net charge of −5 at pH 7.4 [23] – should experience relatively less electrostatic destabilization than the rat β-PV, which has a predicted net charge of −16 [33]. Moreover, the additional residue in the α-PV F helix enables the C-terminal carboxylate to form H-bonds and salt bridges with three residues in the B/C region of the molecule – Lys-27, Gln-31, and Lys-36 – in the Ca^{2+}-bound protein. Truncation of the β-PV helix at residue 108 effectively eliminates these tertiary interactions. The structural differences at the C-terminus are indicated in Fig. 5.

Although they were obtained on the Ca^{2+}-loaded α- and β-parvalbumins, the structures depicted in Fig. 5 are relevant to the apo-proteins as well. The Ca^{2+}-free macrostate is, in fact, an ensemble of conformational microstates, each of which contributes to the thermodynamic properties of the apo-protein. UV absorbance, circular dichroism, and fluorescence data suggest that the *average* apo- and Ca^{2+}-bound structures are perceptibly different (e.g. [36–38]). However, NMR data unequivocally demonstrate that a highly ordered conformation resembling the predominant Ca^{2+}-bound conformation is included – and significantly populated – in the apo-PV ensemble. Williams et al. [32] estimate that 85% of the rat apo-α-PV molecules reside in this conformation at 25°C. With respect to the rat β-PV, we find that the majority of amide proton resonances for residues in the AB domain and E helix display virtually identical chemical shifts in the ^{15}N-HSQC spectra of the apo- and Ca^{2+}-bound forms (J.J. Likos and M.T. Henzl, unpublished observations).

Despite its much more negative charge and truncated F helix, the β isoform exhibits perceptibly greater thermal stability at physiological pH and ionic strength. Whereas the α-PV displays a T_m of 45.8°C in 0.20 M NaCl, 0.01 M EDTA, pH 7.4, the β-PV denatures at 53.6°C. The physical basis for the heightened stability of the β isoform is currently conjectural. Clearly, however, the additional residue in the α-PV C-terminal helix is not the predominant determinant of PV conformational stability.

Paradoxically, the heightened stability of the rat β-PV may have an electrostatic origin. Tanner et al. [39] noted that the stabilities of glyceraldehyde 3-phosphate dehydrogenase from thermophilic and mesophilic organisms correlate well with the number of hydrogen bonds between charged side chains and neutral partners. These charged-neutral hydrogen bonds may well be more numerous in the β isoform (25 acidic residues, 9 basic) than in the α (22 acidic, 17 basic). Alternatively, the β-PV could be stabilized by strategic placement of salt bridges. Although ion pairs are generally believed to exert modest influence on protein stability (e.g. [40–42]), due to the desolvation penalty that accompanies ion-pair formation, the existing data suggest that select proteins are stabilized by ionic interactions [43–45]. Finally, local and/or global charge differences between the two PV isoforms could produce differential interactions with solvent ions. The linkage between ion binding and protein folding/stability is an active area of investigation [46,47]. Efforts to distinguish between these three potential explanations are currently in progress.

Although not the overriding determinant of conformational stability, the additional residue in the α-PV C-terminal helix nevertheless exerts a significant stabilizing influence. The predicted conformational stability of the apo-α-PV at 25°C is just 3.5 kcal/mol. Truncation of the F helix – by removal of the penultimate residue, glu-108 – reduces ΔG_{conf} by 0.9 kcal/mol, or roughly 25%. Presumably, this effect reflects the elimination of favorable non-covalent interactions between the C-terminal carboxylate and residues 27, 31, and 36. We hasten to add, however, that the removal of Glu-108 – conserved among all α isoforms – may also eliminate a stabilizing salt bridge between the side chain carboxylate of Glu-108 and the ε-ammonium group of Lys-27. Corson et al. [48] alluded to the potential significance of this interaction in their detailed study on the consequences of stepwise proteolysis in carp (p*I* 4.25) parvalbumin. The characterization of the α-PV Δ109S variant – in which truncation is achieved by simply removing Ser-109 – could help clarify this issue.

In this context, it is significant that extension of the β-PV F helix, by addition of Ser-109, did not cause a similarly profound stabilization of the β-PV. There are at least two potential explanations for the minimal impact of this mutation. Although extension of the F helix may introduce stabilizing interactions between the Ser-109 carboxylate and residues 27, 31, and 36 in the B/C region, it conceivably eliminates a favorable hydrogen bond between the C-terminal carboxylate of residue 108 and Gln-27. In fact, as noted by Corson et al. [48], the α-PVs may have incorporated an acidic glutamyl side chain at position 108 in the interest of preserving this stabilizing interaction. Additionally, the residues in the B/C region of the β-PV may not be optimized for the interaction with the C-terminus of the extended F helix. For example, glutamine and serine replace the lysines that occupy positions 27 and 36 in the α isoform. It will be interesting to ascertain the consequences of simultaneously extending the F-helix of the β-PV and introducing the Q27K and/or S36K mutations.

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