Results: Intrinsic Fluorescence in Unfolding

Thermal denaturation of the α1-5 constructs was also assessed by tryptophan fluorescence. This provides an indirect measure of tertiary structure changes that relate to hydration of these hydrophobically buried aromatic side chains (35, 36). The WT exhibits two major transitions (Supp. Fig. 1a), in qualitative agreement with the CD results. The first change in slope corresponds to a structural transition at $T_{mI} \approx 45$ °C from Trp fluorescence *versus* a mid-transition of 48 °C from DSC and CD. The second transition is at $T_{mII} \approx 56$ °C *versus* a mid-transition of 59-60 °C from DSC and CD. For the WT, the signal changes at each transition are nearly equal (0.6 and 0.4) and in reasonable proportion to the equal number of Trp in α1-3 and α4-5 (5 in each). In contrast, for the Q471P mutant, the dramatic decrease at a single transition near 37 °C suggests that the proline mutation in the linker destabilizes the entire 5 repeat construct. The CD results above for the mutant indeed showed that the second apparent transition is also shifted to lower temperature (Fig. 2a).

Fluorescence Spectroscopy Method

All tryptophan fluorescence experiments were carried out using a UV spectofluorometer from Photon Technology Instruments with a set excitation wavelength of 275 nm. The emission spectrum was monitored between 310-370 nm for each scan. Samples consisted of 480 µL of PBS (pH = 7.5) and 20 uL of 1mg/mL protein utilizing a 1mL cuvette with 1cm path length. Temperature scans from 21 to 80 degrees were collected at 2-degree increments with appropriate time to thermoequilibriate at each temperature. A blank buffer sample spectra was also collected for background subtraction. The emission intensities at 350nm were analyzed using the previously reported method of Pace (19).

Analysis: Fit of erythrocyte spectrin tetramerization

The thermodynamic equilibrium between the dimer (*D*) and tetramer (*T*) forms of spectrin can be expressed as

$$K_a = T / D^2 \qquad \text{(S1)}.$$

Since the plot of the dimer/tetramer ratio asymptotes with a significant amount of dimer still in solution, we conclude that there is a fraction *d* of the total concentration of spectrin *S*, that is not capable of associating into the tetrameric form. Mass conservation is thus $S = D + d + T$. Rearrangement gives $D = S - d - T$, and allows for the association constant K_a to be written as

$$K_a (S - d - T)^2 - T = 0 \qquad \text{(S2)}$$

Given that $K_d = 1/K_a$ then substitution of K_a results in

$$d^2 + T^2 + S^2 + 2dT - 2dS - 2TS - TK_d = 0 \qquad \text{(S3)}$$

Dividing by S^2 yields

$$(T/S)^2 - \frac{T}{S}\left(-2\frac{d}{S} + 2 + \frac{K_d}{S}\right) + \left[1 - 2\frac{d}{S} + \left(\frac{d}{S}\right)^2\right] = 0 \qquad \text{(S4)}$$

Solving for *T/S* and assuming *d/S* is a constant fraction, denoted by *f*

$$2\frac{T}{S} = \frac{K_d}{S} + 2(1-f) \pm \left[\left(\frac{K_d}{S} + 2(1-f)\right)^2 - 4(1-f)^2\right]^{1/2} \qquad \text{(S5)}$$

for which the negative sign gives physically meaningful results.

In the limit $S >> K_d$, $(T/S) = 1 - f$.

Note that Eq. 5 can be substituted into the conservation of mass expression to give

$$(D + d) / T = -1 + (T / S)^{-1} \qquad \text{(S6)}$$

Applying Eq. 6 to the data of Fig. 1b yields a $K_d = 0.62$ μM ($R^2 = 0.98$) for the wild-type spectrin control, and $K_d = 1.1$μM ($R^2 = 0.99$) for Q471P mutant.