Site-directed Mutagenesis of Either the Highly Conserved Trp-22 or the Moderately Conserved Trp-95 to a Large, Hydrophobic Residue Reduces the Thermodynamic Stability of a Spectrin Repeating Unit*

(Received for publication, January 24, 1997, and in revised form, May 24, 1997)

Dennis P. Pantazatos and Ruby I. MacDonald†
From the Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208

As reported previously (MacDonald, R. I., Musacchio, A., Holmgren, R. A., and Saraste, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1299–1303), an unfolded peptide was obtained by site-directed mutagenesis of Trp-22 to Ala in the cloned, wild type 17th repeating unit (α17) of chicken brain α-spectrin. Trp occurs in position 22 of nearly all repeating units of spectrin. In the present study, Trp-22 was mutated to Phe or to Tyr to compare thermodynamic stabilities of urea-induced unfolding of α16 and mutants thereof. α16 was chosen for this study instead of α17, because α16 has two tryptophans, allowing urea-induced unfolding to be tracked by the fluorescence of the Trp remaining in each mutant peptide and by circular dichroism in the far UV.

The free energies of unfolding of W22Y and W22F were 50% that of α16, showing that Trp-22 is crucial in stabilizing the triple helical bundle motif of the spectrin repeating unit. Mutation of the moderately conserved Trp-95 of α16 to Val, which occupies position 95 in α17, also yielded a peptide with 50% of the free energy of unfolding of α16. Thus, the thermodynamic stability of a given spectrin repeating unit may depend on both moderately and highly conserved tryptophans. Different structural roles of Trp-22 and Trp-95 in α16 are suggested by the slightly higher wavelength of maximum emission of Trp-22, the greater acrylamide quenching of Trp-95 than Trp-22, and the longer lifetime of Trp-95. For comparison with α16, urea-induced unfolding of spectrin dimer isolated from human red cells was monitored by far UV-CD and by tryptophan fluorescence. Thermodynamic parameters could not be rigorously derived for the stability of spectrin dimer because unfolding of spectrin dimer involved more than two states, unlike unfolding of cloned repeating units. However, the similar midpoints of CD-monitored denaturation curves of α16 and spectrin dimer, i.e. 2.7 and 3.2 M urea, respectively, indicate that investigation of cloned repeating units of spectrin can provide physiologically relevant information on these structures.

The flexibility of the cytoskeletal protein spectrin is due to domains of 20 (α subunit) and 17 (β subunit), linearly connected repeating units, each of which is folded into a triple-helical bundle (1, 2). It should be noted that the widely accepted term “repeating unit” does not imply sequence homology, since only one to two dozen amino acids are highly conserved among the 106–119 residues of these units of spectrin (3), as well as those of the closely related α-actinin (4) and dystrophin (5). Mutations of some of these conserved residues have pathologic consequences in organisms as diverse as Drosophila (6) and man (3, 7), although mutations of nonconserved residues of at least human erythroid spectrin have also been associated with certain hemolytic anemias (3, 7). These findings warrant further investigation of the relationship between the sequences of the repeating units and their biochemical and biophysical properties. A promising approach is the study of cloned repeating units that can be selectively mutated, as well as expressed singly or in numbers optimal for the measurement and analysis of those biochemical and biophysical properties. The feasibility of cloning one or more repeating units was first demonstrated by Winograd et al. (8) who obtained repeating units in a native-like conformation only when the spectrin cDNA coded for an integral number of repeats, i.e. when the cDNA was “conformationally phased.”

Our interest is focussed on the tryptophan that is nearly invariant among the repeating units of spectrin (3), α-actinin (4) and dystrophin (5). In x-ray crystallography (9) and NMR (10) structures of two different triple helical, single repeating units of spectrin, Trp-22 is located in a site between the bulk phase and the region bounded by the three α helices. This partially shielded position of Trp-22 is also indicated by its blue-shifted wavelength of maximum emission in α17, the cloned 17th repeating unit of chicken brain α-spectrin (11). To probe the role of the highly conserved Trp-22 in the folding of the 17th repeating unit of chicken brain α-spectrin (11), we mutated Trp-22 to alanine and found that α17 W22A was unfolded, suggesting that Trp-22 promotes stable folding of a repeating unit (11).

More conservative mutations of Trp-22 to Phe or to Tyr were made in the present study to obtain folded Trp-22 mutants for quantitative measurement of the importance of Trp-22 for the thermodynamic stability of a spectrin repeating unit. The 16th repeating unit (α16) of chicken brain α-spectrin was chosen for the present study instead of α17, because α16 has two tryptophans instead of the single one in α17. Hence, the fluorescence of the tryptophan remaining after site-directed mutagenesis of either the highly conserved Trp-22 or the less highly conserved Trp-95 could be measured to monitor tertiary structure during urea-induced unfolding of the mutant peptides. Secondary structure was monitored during urea-induced unfolding by CD at 222 nm. The free energies of unfolding of mutant peptides W22F and W22Y were 50% of α16, establishing that Trp-22 significantly stabilizes the folding of α16.

A major contribution to understanding how folding of the repeating unit is stabilized has been made by modeling the amino acid sequences of the helical regions of repeating units of
Thermodynamic Stability of Mutant Spectrin Repeats

was prepared by oligonucleotide-directed PCR of chicken brain α-spectrin cDNA (15) by AmpliTaq polymerase (Perkin-Elmer). Mutations W22Y, W22F, and W95V were introduced in oligonucleotides, as described previously (11). Amplified DNA fragments were isolated from agarose gels, ligated to pETSc vector (16), and used to transform BL21(DE3) strain Escherichia coli, made competent by treatment with 

RbCl (17). Transformed cells were selected by growth on ampicillin plates. The sequences of inserted cDNAs of all plasmids were verified by DNA sequencing and are shown in Table I. The sequence of α16 is as described previously (11), except that alanine is added at the N terminus which then is A and not K, and is exactly the same as the corresponding sequence from chicken brain α-spectrin (15).

Expression and Purification of Peptides—The peptides were obtained by growing plasmid-containing cells in 3 liters of Luria-Bertani medium + 100 μg/ml ampicillin at 37 °C until the A600 nm was between 0.2 and 0.6. Isopropyl β-D-thiogalactoside was added to a concentration of 0.5 mM and growth continued for another 3 h. After pelleting in the JS-4.2 rotor of a Beckman J-6.0 centrifuge at 3,297 × g for 1 h and storage at −20 °C, the cells were sonicated in about 10 volumes of chilled, 0.02 mM Tris-HCl, pH 8, + 1 mM EDTA + 0.15 mM phenylmethylsulfonyl fluoride + 1 mM dithiothreitol for 5, 1-min sonication periods alternating with 1-min rest periods. Cell debris was removed at 4 °C by ultracentrifugation in a type 45T rotor for 1 h at 186,000 × g or by centrifugation in a Sorvall SS34 rotor for 1 h at 27,000 × g. The supernatant was passed through a 0.45-μm filter and a 0.25-μm filter prior to desalting on a 5PW high performance liquid chromatography (Toso-Haas). Peptides were detected on 15% SDS-PAGE gels in 3 μl urea, further purified on Sephacryl S-100 (Sigma) and/or Q-Sepharose (Sigma) prior to desalting on 10DG columns (Bio-Rad) and stored at −20 °C in 10 mM sodium phosphate, pH 8. Peptides were at least 99% pure (with the exception of W22F which was at least 97% pure) by densitometry of SDS-PAGE gels.

Yields were 10–100 mg per 3 liters of culture.

Peptide concentrations were determined from their absorbance at 280 nm, their tryptophan and tyrosine contents and the molar extinction coefficients at 280 nm for tryptophan and tyrosine (11). Exposure of the peptides to 6 M guanidine hydrochloride for comparison with native peptides (19) had a negligible (<5%, except for α16 W22F at 8%) effect on their absorbance. The first 10 amino acids at the N terminus of the wild type, 16th repeating unit (α16, formerly R16 (11)), were sequenced by Dr. Joseph Leykam at the Macromolecular Structure Facility, Michigan State University, and found to be the same as in Table I. Mass spectrometry analysis of all six peptides by Dr. Richard Milberg at the University of Illinois, Urbana, gave the expected M+ values 0.1–0.2%.

According to published methods (20), a crude extract of spectrin was obtained by incubating red cell ghosts, which had been lysed from freshly drawn human red cells, in 0.5 mM β-mercaptoethanol + 0.02 mM diisopropyl fluorophosphate + 0.1 mM EDTA, pH 9, at 37 °C. The crude extract was concentrated by ultrafiltration and layered on a Sepharose CL-4B column to isolate spectrin dimers. The spectrin dimer concentration was calculated from its extinction coefficient at 280 nm, i.e. 10.0 for a 1% solution (20). Densitometry of spectrin analyzed by SDS-PAGE on a polyacrylamide gel showed it to be at least 99% pure.

Fluorescence Measurements—8 or 10 μl stock solutions of urea were made on the day of use by dissolving the urea in 10 mM sodium phosphate, pH 7.4, to maintain a pH of 8 in the peptide-containing samples. For Stern-Volmer measurements 1 mM acrylamide stock solutions were also prepared on the day of use. All samples gave an absorbance at 295 nm of less than 0.1 in a cell of 1 cm path length so that correction for an inner filter effect was unnecessary (21). Steady-state fluorescence of 3 to 5 μM peptide or 50 μg/ml spectrin dimer in a cell of 0.5 cm path length was measured at room temperature with an Alpha-Scan fluorimeter (Photon Technology International) at a 4 or 6 nm band pass. Peptidyl tryptophan was excited at 295 nm and generally scanned from 300 to 400 nm. Raman and Rayleigh signals were subtracted from each scan. Measurements of urea-containing samples were performed at least 1 h after additions of the peptide to solutions at the appropriate urea concentrations. After scanning, the samples were usually stored at 4 °C overnight and re-scanned the next day to detect any changes in the recordings that might alter the evaluation of thermodynamic parameters of unfolding. As no such changes were detected, all samples appeared to have attained equilibrium after an hour at room temperature. Fluorescence lifetimes were measured at room temperature in a LS180, nanosecond pulse fluorometer (Photon Technology International) with a hydrogen lamp in the time-correlated, single photon counting mode. Samples in a cell of 0.5 cm path length were excited at 297 nm and the emission recorded at 330 nm. To correct for the excitation pulse, light scattering of a second sample of 0.1 mg/ml glycogen was measured before each excitation of the peptide sample. Decays were fit to one or more components.
Thermodynamic Stability of Mutant Spectrin Repeats

more exponentials by an iterative procedure based on the Marquardt algorithm (22). $x^2$ values ranged from 0.9 to 1.2.

Circular Dichroism Measurements—Far-UV CD spectra of peptides at 5 μM in 10 mM sodium phosphate, pH 8, or of spectrin dimer at 50 μg/ml in 0.1 M NaCl + 0.01 M Tris-HCl, pH 7.5, + 0.1 mM EDTA + 0.1 mM β-mercaptoethanol in a 0.1-cm quartz cell were taken at room temperature with a Jasco 500C spectropolarimeter. Calibration was performed with a solution of d-10-camphorsulfonic acid (23), and base lines were subtracted from all samples. CD measurements are reported as mean residue ellipticities, [θ], in degrees cm$^2$/dmol. Spectra were scanned from 250 to 190 nm in 1-nm steps with a 4-s time constant at a rate of 20 nm/min. The photomultiplier voltages corresponding with the reported values were less than 500 V.

Calculation of $m$, $U_{50\%}$, and $\Delta G^{H2O}_{UN}$—We followed the linear extrapolation method (24), as modified (25) to include nonlinear fitting of data to Equation 1 below, which has been evaluated recently with simulated fluorescence data (26). Application of this method has given the same thermodynamic values for unfolding (UN) of model peptides regardless of the mechanism assumed for solute-induced denaturation, i.e. denaturant binding or solvent transfer (27). This method has also yielded the same $\Delta G^{H2O}_{UN}$ values of thioredoxin unfolding when induced either by thermal or by solute denaturation (28) and the same $\Delta G^{H2O}_{UN}$ values of RNase unfolding whether induced by urea or by guanidine hydrochloride (29).

Our data for the cloned peptides met the requirements for analysis by this method, since measurements were made at equilibrium and urea-induced unfolding of cloned repeating units was reversible (Figs. 2 and 4) and consisted of two states with neither detectable intermediates (Fig. 5) nor significant aggregation of the native state (Fig. 4). In the case of spectrin dimer, however, plots of fluorescence intensity at a single wavelength did not yield a smooth urea denaturation curve (not shown), apparently due to nonsimultaneous unfolding of its many repeating units, as well as nonhomologous domains. However, calculation of an intensity-averaged emission wavelength, $\lambda$, for spectrin dimer, according to Ref. 30, plotted versus urea concentration yielded a smooth curve.

Equation 1 (25) was solved for $m$, which is the slope of the transition of denaturation, and $U_{50\%}$, which is the urea concentration at 50% denaturation, from fluorescence (F) or $[\theta]_{222}$ nm values. The product of $m$ and $U_{50\%}$ is the free energy of unfolding in the absence of urea, $\Delta G_{UN}^{H2O}$.

$$F = [(\alpha_1 + \beta_0 U) + (\alpha_0 + \beta_0 U) \exp(m U - m U_{50\%}/RT)}/
(1 + \exp(m U - m U_{50\%}/RT)) \quad (Eq. 1)$$

$\alpha$ is the $y$ intercept of the native (N) or denatured (D) state, $\beta$ is the slope of the native (N) or denatured (D) state, $U$ is the urea concentration, $R$ is the gas constant, 1.987 kcal mol$^{-1}$ K$^{-1}$, and $T$ is the temperature in K.

To superimpose fluorescence and CD denaturation curves in Figs. 5 and 8, the fluorescence intensities and $[\theta]_{222}$ nm from each experiment were converted into values corresponding to fractions of unfolded peptide. Fluorescence intensities at 320 or 330 nm (F), $[\theta]_{222}$ nm, or fraction unfolded values based on fluorescence or CD measurements from two or more experiments for each peptide were pooled and analyzed by nonlinear regression with SigmaPlot 5.0 (Jandel Scientific).

90° Light Scattering—To determine the molecular masses of peptides under the conditions of fluorescence and CD measurement, peptides at 0.05 to 0.5 mg/ml in 10 mM sodium phosphate, pH 8, were excited at 300 or 400 nm in a cell of 0.5 cm path length, whereas light scattering was recorded at 300 or 400 nm and a band pass of 6 or 4 nm, respectively, in an AlphaScan fluorimeter. Standards were bovine serum albumin ($M_r 66,000$) and pancreatic RNase A ($M_r 13,700$), the diameters of which are small enough compared with the wavelength of the exciting light that only molecular size, but not shape, affect the signal (31). The least squares method was used to fit light scattering versus peptide concentration to a curve, the slope of which gave the molecular mass of the peptide by interpolation between the standards by the method of Lagrange (32).

RESULTS

Sequences of Constructs—Amino acid sequences of the six peptides cloned for this study are given in Table I. The peptides are 1) the 16th repeating unit ($\alpha 16$) of chicken brain α-spectrin, 2) the W22Y mutation of $\alpha 16$, 3) the W22F mutation of $\alpha 16$, 4) W22Y with an artificial second mutation, S74L, 5) W22Y

| Table I |
| Amino acid sequences of the 16th repeating unit ($\alpha 16$) of chicken brain α-spectrin and the peptides produced for this study |
| --- |
| Hyphens indicate unchanged residues. The repeating heptad pattern, a through g, of each $\alpha$-helix, which helps to stabilize the triple-helical bundle (12), is indicated as shown (9, 10). |
| **a**16 | gabcdefgabcdefgabcdefgabcdef | abcdgababcd |
| W22Y | A | A | A | A | A |
| W22F | E | E | E |
| W22Y,S74L | F | F | F |
| W22Y,V92M | G | G |
| W95V | Y |
| 60 | 70 | 80 | 90 | 100 | 110 |
| **a**16 | gabcdefgabcdefgabcdef | abcdgababcd |
| W22Y | E | E | E | E | E | E |
| W22F | F | F | F |
| W22Y,S74L | G | G | G |
| W22Y,V92M | H | H |
| W95V | I | I |
| FIG.2. Tryptophan emission spectra of spectrin repeating units were recorded at 3 or 5 μM in 10 mM sodium phosphate, pH 8, but the fluorescence intensities of all peptides have been adjusted to represent 3 μM peptide to facilitate their comparison. The ordinate for a16 is at the upper left; the ordinate for W22Y, W22F, W22Y,S74L and W22Y,V92M is at the upper right and lower left, and the ordinate for W95V is at the lower right. Samples were excited at 295 nm. Solid lines indicate native peptide, lines of dashes indicate peptide unfolded in 4–5 M urea, and lines of dash-dot-dashes indicate peptide unfolded in 4–5 M urea and diluted in 10 mM sodium phosphate, pH 8, to promote refolding. |
with another artifactual second mutation, V92M, and 6) the W95V mutation of α16. The peptides mutated at Trp-22 were cloned to assess its role in the stable folding of α16, and W95V was cloned to compare the fluorescence properties of Trp-22 and Trp-95 and to probe the role of Trp-95 in the stable folding of α16.

Tryptophan Fluorescence Spectra Indicate Reversible Unfolding of All Peptides in Urea—Fig. 2 contains the tryptophan emission spectra of the peptides listed in Table I. To obtain the fluorescence data, 2 aliquots of each peptide were incubated at room temperature in 4 W22F (○) and 16 or 5 M urea (W22Y; W22Y,S74L; W22Y,V92M; W95V) for at least 1 h, after which each sample was diluted 10-fold with 4 or 5 M urea (Fig. 2, dashed line) or with buffer alone (Fig. 2, dash-dot-dashed line). As a control, a 3rd aliquot was incubated simultaneously in buffer alone and subsequently diluted 10-fold with the same buffer (solid line). The reversibility of unfolding of all peptides is shown by the coincidence of scans of native peptides with scans of re-folded peptides. In addition, the more intense fluorescence of Trp-95 in peptides with mutations of Trp-22 compared with the quenched fluorescence of Trp-22 in the peptide with mutated Trp-95 is striking, particularly since the emission maxima of these peptides are similar. Trp-95 in peptides with mutations of Trp-22 compared with the quenched fluorescence of Trp-95 in peptides with mutations of Trp-22 is red-shifted to 354 nm, indicating its shielding from the bulk phase. Trp-22 in W95V is slightly less shielded than Trp-95 with a λ_{max} of 333 ± 0.0 nm in folded W95V and 334.8 ± 2.4 nm in refolded W95V. The λ_{max} of all unfolded peptides is red-shifted to 354 nm, indicating exposure of tryptophan(s) to the bulk phase.

Trp-22 Exhibits a Lower K_{SV} of Acrylamide Quenching Than Trp-95—Acyrlamide quenching of native peptides was performed to determine the accessibilities of Trp-22 and Trp-95 to maintain the same peptide concentration in the case of the native peptide to or to maintain the same peptide concentration in the case of the native peptide. The spectra of refolded W22Y; W22Y,V92M, and W95V were increased by 2,000–3,000 degrees-cm^{-1}dmol^{-1} to make them distinguishable from the spectra of the native forms of those native peptides.

Reversibility of Urea Denaturation of All Peptides Is Also Shown by CD Data—CD spectra with about twice the positive signal at 190 nm as negative signal at 222 nm in Fig. 4 are typical of peptides with largely α-helical structure (34). The spectrum of each peptide in its native form (Fig. 4, solid line), furthermore, overlaps that of the same peptide which had been denatured in 4 M urea and subsequently refolded by dilution and removal of urea on a desalting column (Fig. 4, dashed line). This correspondence of the CD spectra of the untreated (solid line) and urea-treated but refolded (dashed line) forms of each peptide in Fig. 4 corroborates the fluorescence-monitored reversibility of urea denaturation in Fig. 2. Based on the mean residue ellipticity of a peptide with 100% α-helical structure, i.e. 36,000 degrees-cm^{-1}dmol^{-1} at 222 nm (34), the % α-helicity of each peptide was calculated to be 56.5% for α16, 28.4% for W22Y, 39.9% for W22F, 47.6% for W22Y,S74L, 40.4% for W22Y,V92M and 25.3% for W95V. The 56.6% value for α16 is within the range of values obtained for α16, averaging 66.7% ± 12.9. The helical content of each peptide is roughly commensurate with its free energy of peptide unfolding given in Fig. 6.

Urea Denaturation Curves Based on Fluorescence and CD Data Are Superimposable for All Peptides—Given that the conditions for thermodynamic analysis of the stability of peptide folding were satisfied, peptide unfolding was monitored at increasing concentrations of urea. Tryptophan fluorescence and ellipticity at 222 nm of urea-treated peptides were converted into fractions of unfolded peptide, plotted versus urea concentration, and fit to Equation 1 under “Experimental Procedures” describing peptide unfolding as a two-state, reversible process at equilibrium. In Fig. 5 urea denaturation curves based on tryptophan fluorescence (Fig. 5, open circles) coincide with urea denaturation curves based on the mean residue ellipticity at 222 nm (Fig. 5, solid circles) for all peptides with the possible exception of W22Y,S74L. The thermodynamic parameters from fluorescence and CD data in Fig. 6, however, are identical.
within standard error for all peptides, so that unfolding appears not to involve intermediate states (35).

Thermodynamic Parameters of Peptide Unfolding—Values for thermodynamic parameters from data in Fig. 5 are given in Fig. 6. The $U_{50\%}$, representing the average of fluorescence and CD values range from 2.44 M urea for W22Y,S74L, 2.38 M urea for $\alpha_{16}$, 1.77 M urea for W22Y,V92M, 1.38 M urea for W22Y, 1.37 M urea for W22F to 1.04 M urea for W95V. In contrast with these $U_{50\%}$ values, the cooperativity of unfolding, $m$, ± S.E. is about the same for all peptides, with the possible exception of W22Y. Hence, the product of $U_{50\%}$ and $m$, $\Delta G^\text{HEL}$, ranges nearly in parallel with the $U_{50\%}$ values from 4.5 kcal/mol for $\alpha_{16}$, 4.3 kcal/mol for W22Y,S74L, 2.9 kcal/mol for W22Y,V92M, 2.3 kcal/mol for W22F, 2.0 kcal/mol for W22Y to 1.9 kcal/mol for W95V. Thus, at 25 °C and in 10 mM sodium phosphate, pH 8, the artificial, second mutation in W22Y,V92M partially restores and in W22Y,S74L completely restores the significantly diminished stability of folding due to substitution of the highly conserved tryptophan Trp-22 with tyrosine.

Urea Denaturation Curves of Wild Type and W22Y,S74L Under More Physiological Conditions—We next examined the similarly stable $\alpha_{16}$ and W22Y,S74L under more physiological conditions of ionic strength and temperature to assess whether a second mutation like S74L might compensate for the destabilizing effect of a mutation like W22Y in vivo and to enable comparison with the unfolding of intact spectrin dimer. Data in Fig. 7A were obtained at 25 °C and data in Fig. 7B were obtained at 37 °C, both in 0.14 M KCl + 10 mM sodium phosphate, pH 7.5. From their $\Delta G^\text{HEL}$ values in Table II, W22Y,S74L and the wild type are still equally stably folded at 25 °C and more stably folded than W22F, but the wild type is marginally more stably folded than W22Y,S74L at 37 °C.

Urea Denaturation of Spectrin Dimer Isolated from Human Red Cells—For comparison with urea-induced unfolding of $\alpha_{16}$ in Fig. 7A, spectrin dimer was isolated from human red cells and its urea-induced denaturation followed by $\theta_{222}$ and by tryptophan fluorescence in 0.1 M NaCl + 10 mM Tris-HCl, pH 7.5, + 0.1 mM EDTA + 0.1 mM β-mercaptoethanol. Nearly identical with the average 66.7% α-helical content of $\alpha_{16}$, the α-helical content of intact spectrin dimer was 68.2%, based on a 100% value of 36,000 degrees cm$^2$ mol$^{-1}$ (34). Both of the fraction unfolded curves in Fig. 8, one based on $\theta_{222}$ and the other on the intensity-averaged emission wavelength, appear to describe a two-state transition of spectrin dimer unfolding. However, calculation of a putative $[U]_{50\%}$ yielded 3.2 ± 0.69 M urea for the CD curve and 4.4 ± 0.68 M urea for the fluorescence curve, and calculation of a putative m yielded 0.29 ± 0.16 kcal/mol/m for the CD curve and 0.38 ± 0.12 kcal/mol/m for the fluorescence curve. Comparison of these values for spectrin dimer with those for the cloned peptides in Table II and Fig. 6 reveals the standard error of these values for spectrin dimer to be about an order of magnitude larger than for the well-behaved $[U]_{50\%}$ and m of the cloned peptides. Also, the 1.2 M difference in $[U]_{50\%}$ from CD versus fluorescence data of intact spectrin dimer is much greater than the <0.2 M difference in $[U]_{50\%}$ from CD versus fluorescence data of the cloned $\alpha_{16}$ and mutant peptides. Finally, the values for m of spectrin dimer are very low, about 1/6 that of m for the cloned peptides. Thus, in
TABLE II
Free energies of peptide unfolding induced by urea in 0.14 M KCl at pH 7.5 and monitored by tryptophan fluorescence

| Peptide | m | U_{50\%} | \Delta G_{UN} |
|---------|---|----------|-------------|
| \alpha 16 (25 °C) | 1.75 ± 0.19 | 2.73 ± 0.05 | 4.77 ± 0.52 |
| W22F (25 °C) | 1.92 ± 0.15 | 1.91 ± 0.04 | 3.66 ± 0.35 |
| W22Y,S74L (25 °C) | 1.61 ± 0.24 | 2.81 ± 0.07 | 4.54 ± 0.67 |
| \alpha 16 (37 °C) | 1.58 ± 0.21 | 1.89 ± 0.09 | 2.99 ± 0.49 |
| W22Y,S74L (37 °C) | 1.38 ± 0.09 | 1.50 ± 0.07 | 2.07 ± 0.23 |

* Temperature at which the measurement was made is given in parentheses.

Fig. 8. Denaturation curves of fraction of spectrin dimer unfolded versus urea concentration at room temperature, calculated from the measured \(\Theta_{222}^M\) (○) and from the intensity-averaged emission wavelength, \(\lambda\) (○). The dimer concentration is 50 \(\mu\text{g} / \text{ml}\) in 0.1 M NaCl + 10 mM Tris, pH 7.5, + 0.1 mM EDTA + 0.1 mM \(\beta\)-mercaptoethanol.

Contrast with the authentically two-state denaturation curves of cloned peptides in Fig. 5, the resemblance to a two-state transition of denaturation curves of spectrin dimer in Fig. 8 is misleading.

All Peptides Except for W22F Are Monomers at the Outset of Urea-induced Unfolding. According to 90° Light Scattering—Fig. 9 is a plot of 90° light scattering versus increasing concentrations of all six peptides to determine their molecular masses in solution. Data in the upper 3 panels were obtained at a band pass of 6 nm with the excitation and emission monochromators set at 300 nm, whereas data in the lower 3 panels were obtained at a band pass of 4 nm with the excitation and emission monochromators set at 400 nm. Standards are represented in each panel by the upper dashed line for bovine serum albumin \((M, 66,000)\) shown without data points and the lower dashed line for RNase A \((M, 13,700)\) also shown without data points. The following masses ± S.E. were determined: 13,770 ± 1,628 for \(\alpha 16\); 19,244 ± 1,922 for W22F; 12,927 ± 1,098 for W22Y,S74L; 11,681 ± 253 for W22Y; 12,315 ± 1,114 for W22Y,V92M, and 14,040 ± 240 for W95V. As expected, these molecular masses obtained by light scattering are more variable and less accurate than those obtained by mass spectrometry (see "Experimental Procedures") but were necessary to assess the degree of aggregation of the peptides under the conditions of the fluorescence and CD measurements. \(\alpha 16\) has also been shown by Pascual et al. (10) to be a monomer by analytical ultracentrifugation, unlike the wild type, 14th repeating unit of Drosophila \(\alpha\)-spectrin (36).

Although the W22F mutant peptide was not monomeric in its native state, its CD and fluorescence-based denaturation curves in Fig. 5 appear well-behaved in terms of 1) the coincidence of these curves, 2) the identity of thermodynamic parameters (± S.E.) obtained from these curves, and 3) the general appearance of these curves indicating that W22F unfolding is a two-state process. Thus, we have established that thermodynamic analysis of urea-induced unfolding of cloned repeating units is valid since peptide unfolding was reversible (Figs. 2 and 4), data were taken at equilibrium, unfolding involves two states from the sigmoidal and superimposable far UV-CD and tryptophan fluorescence denaturation curves (Fig. 5), and all peptides with the exception of W22F were monomers by 90° light scattering (Fig. 9).

DISCUSSION

Conservative Mutation of the Nearly Invariant Trp-22 Significantly Reduces the Thermodynamic Stability of Folding of a Repeating Unit of Spectrin—Mutation of the nearly invariant Trp-22 to tyrosine or to phenylalanine, which are most frequently substituted for tryptophan in nature (37), reduces the \(\Delta G_{UN}\) of \(\alpha 16\) of chicken brain \(\alpha\)-spectrin by 50% (Fig. 6). Hence, even amino acids most closely resembling tryptophan in size and hydrophobicity fail to substitute for Trp-22 in maintaining the stability of folding of a wild type repeating unit of spectrin. As noted in the Introduction and indicated in Table I, Trp-22 does not occupy an a or d position in the repeating heptad pattern (Fig. 1), where stabilization of the triple helical bundle of the repeating unit may occur through hydrophobic interactions (12). Contrary to expectations for a large, hydrophobic amino acid, Trp-22 occupies a g position (Table I), where stabilization may occur through the formation of salt bridges (12). Furthermore, Trp-22 is unique in chicken brain \(\alpha\)-spectrin as a large, hydrophobic amino acid which is highly conserved in a g position of the repeating heptad pattern (10, 15). Thus, the situation of the nearly invariant Trp-22 suggests a third type of mechanism for stabilizing the triple helical bundle motif of a repeating unit of spectrin, in addition to interhelical hydrophobic interactions and salt bridges (12).

Mutations of a Moderately Conserved and Two Nonconserved Residues Also Affect the Free Energy of Unfolding of \(\alpha 16\)—It seemed possible that valine would be an adequate substitute for Trp-95 in \(\alpha 16\), since valine occupies position 95 of \(\alpha 17\) of chicken brain \(\alpha\)-spectrin (15). On the other hand, as indicated in Table I, Trp-95 occurs in the a position of the repeating heptad pattern (9, 10) where hydrophobic interactions could
Thermodynamic Stability of Mutant Spectrin Repeats

impose stringent packing requirements (12). The validity of the latter point is supported by the 50% lower ΔG^\text{UN}_{\text{H}2\text{O}} of W95V than α16. In the crystal structure of the 14th repeating unit of Drosophila α-spectrin (9) hydrogen bonding of Trp-95 and Trp-22 to other residues appears unlikely. Therefore, destabilization resulting from substitution of either Trp-22 or Trp-95, particularly Trp-95 since it occupies an a position in the repeating heptad pattern, may be due to the creation of a cavity left by replacement of the tryptophan with a smaller amino acid. Also possibly indicating the importance of nonconserved amino acids among repeating units for their stability of folding, additional mutations in W22Y of S74L or of V92M, artifactually occurring during PCR, restored the ΔG^\text{UN}_{\text{H}2\text{O}} of W22Y almost entirely or by about 50%, respectively. The greater stability of W22Y, V92M than W22Y can be ascribed to Met providing a more polar interaction than Val at position 92 which is an e position (Table I). In contrast, the Ser → Leu change cannot be rationalized in the same way, since position 74 is also an e position (Table I), which should be less well filled by substituting a polar residue like Ser with a more nonpolar residue such as Leu. It remains to be determined how S74L compensates for the destabilizing effect of W22Y. Whereas it would have been predicted that any substitution of a highly conserved residue such as the nearly invariant Trp-22 should result in destabilization of folding of a cloned repeating unit, it is less certain that mutations of moderately or even nonconserved residues in general would affect the thermodynamic stability of a repeating unit. This question may be particularly relevant to understanding the evolution of spectrin, e.g. by duplication of groups of repeating units (38), and the mechanism underlying hemolytic anemias correlated with single mutations of nonconserved amino acids (3, 7) and deserves further investigation.

Comparison of Urea-induced Denaturation of α16 and W22Y,S74L under More Physiological Conditions—We compared urea-induced unfolding of W22Y,S74L and α16, which exhibited similar ΔG^\text{UN}_{\text{H}2\text{O}} values, in 0.14 M KCl + 10 mM sodium phosphate, pH 7.5, at 25 and 37 °C to assess the ability of S74L to compensate for W22Y under more physiological conditions. As discussed below, these data for α16 were also necessary for comparison with data on the unfolding of spectrin dimer isolated from human red cells (Fig. 8). W22Y,S74L remained as stable as the wild type at 25 °C (Fig. 7A) and was only marginally less stable than the wild type at 37 °C (Fig. 7B). Despite the ability of mutations like S74L to compensate for the destabilizing mutation of Trp-22 in a cloned repeating unit, such secondary mutations are not found in vivo since Trp-22 is so highly conserved among repeating units of spectrin. It may be that the probability of secondary mutations like S74L occurring simultaneously in the same repeating unit as the destabilizing mutation of Trp-22 is extremely low in vivo and/or that Trp-22 is uniquely important in vivo for a second vital function, in addition to stable folding, for which a second mutation cannot compensate.

Comparison of Urea-induced Unfolding of α16 with That of Human Red Cell Spectrin Dimers—Although the CD and fluorescence-based, urea denaturation curves of spectrin dimers in Fig. 8 resemble two-state transitions, the following observations indicate that more than two states are involved: 1) the order of magnitude greater standard errors of the putative [U]_\text{mon} and m of the unfolding of human red cell spectrin dimer than those of the corresponding parameters of α16 unfolding; 2) the much larger difference between the putative [U]_\text{mon} of fluorescence and CD curves for spectrin dimer in Fig. 8 than for the definitive [U]_\text{mon} of fluorescence and CD curves for cloned repeating units in Fig. 5, i.e. 1.2 m versus < 0.3 m, respectively; and 3) the much smaller m of spectrin dimer unfolding compared with the m of α16 unfolding, 0.3 and 0.38 kcal/mol/°M versus 1.75 kcal/mol/°M, respectively. These differences also suggest that the multiple domains of spectrin dimer unfold independently of each other so that the midpoint of the dimer curve may represent the urea concentration at which half of the repeating units, instead of half of each repeating unit, have unfolded. Hence, thermodynamic parameters of unfolding of spectrin dimer were not obtained with the same rigor as thermodynamic parameters of unfolding of cloned, repeating units. Nevertheless, the roughly similar [U]_\text{mon} values calculated from CD-monitored denaturation of α16 and of human red cell spectrin dimer, 2.7 versus 3.4 m, respectively, indicate that the conformation of cloned α16 could reasonably represent its conformation in a spectrin dimer. α-Spectrin of chicken brain is identical to α-spectrin of avian red cells, since avian α-spectrin occurs as a single isoform (39). Thus, it is reasonable to compare α16 of chicken brain α-spectrin with intact spectrin dimer from human red cells, which is more readily obtained than that from chicken red cells.)

The 4.77 ± 0.52 kcal/mol ΔG^\text{UN}_{\text{H}2\text{O}} of unfolding of α16 indicates a greater stability than the 3.4 kcal/mol first reported for a cloned repeating unit of dystrophin, measured by CD of heat or urea-denaturated peptide (40). A subsequent report by the same group (41) gave a significantly higher ΔG^\text{UN}_{\text{H}2\text{O}} of 6.4 kcal/mol but about the same 72% α-helical content for the same repeating unit which had been extended at the C terminus by four residues. Differences in the consensus sequences of spectrin and dystrophin repeating units indicate that their folds may vary significantly despite their origin from a common motif (12, 42). Hence, comment on the reason for the differences between the free energy of unfolding of a spectrin repeating unit reported here and those found for the dystrophin repeating units (40, 41) would be premature. Since the original submission of this manuscript, two reports of similar differences in the unfolding of cloned, repeating units of red cell α-spectrin have come to our attention (43, 44). Further investigation is required to ascertain the structural significance of these differences.

Structural Basis of Differences in Fluorescence Intensities of Trp-22 and Trp-95—Multiple tryptophans in a protein frequently exhibit different quantum yields which change on protein unfolding, e.g. Ref. 30. In the present instance, the structural basis of (i) enhanced fluorescence of Trp-95 in W22F and W22Y, (ii) quenched fluorescence of Trp-22 in W95V, but (iii) quenched fluorescence of both Trp-95 and Trp-22 in α16 (all relative to fluorescence in the unfolded peptides) is apparent in Fig. 10.
Suggested by the structures of Yan et al. (9) and of Pascual et al. (10), Fig. 10 shows that both Trp-22 and Trp-95 lie near histidine 59, a strong tryptophan quencher (45). Pascual et al. (10) have detected nuclear Overhauser effects both between Trp-22 and His-59 and between Trp-95 and His-59. The quenching by histidine 59 of Trp-22 alone but not Trp-95 alone may be due to the different orientations of the two tryptophans to His-59, the smaller of the indole rings of Trp-22 but the larger of the indole rings of Trp-95 being closer to the histidine. The quenching of Trp-95 only when Trp-22 is also present, i.e., in the wild type, probably results from energy transfer from Trp-95 to Trp-22 which is within energy transfer distance. Quenching by histidine of Trp-22, but not Trp-95, in single tryptophan peptides also explains the lower $K_{eq}$ of Trp-22 than of Trp-95 (Fig. 3), since quenching by histidine is expected to shorten the lifetime of Trp-22 alone but not Trp-95 alone. This explanation is compatible with each tryptophan being exposed to the bulk phase to a similar but not identical degree, as affirmed by their wavelengths of maximum emission, i.e., 330 nm for W95V and 334 nm for Trp-22 (Fig. 2).

In summary, the importance of Trp-22 for the thermodynamic stability of a16 and the similarity of the urea concentrations inducing denaturation of 50% of a16 and intact spectrin constitute key evidence that cloned repeating units of spectrin are reasonable models of these structures as they function within cytoskeletal networks. We expect the recombinant DNA approach to continue to further our understanding of how triple-helical repeating units of spectrin are stabilized without mitigating the flexibility of this ubiquitous cytoskeletal protein.

Acknowledgments—We thank Dr. Matti Saraste for the generous gift of chicken brain α-spectrin cDNA, pET8c vector, and primers and, together with Dr. Andrea Musacchio, for introducing us to this system; Dr. Robert Holmgren for advice on cloning; Drs. Theodore Jardetzky and Alfonso Mondragón for providing the ribbon structure in Fig. 10; Steven Abel, Anupama Amaran, Jennifer Dasta, Tracy Irwin, Jennifer Liu, Sri Namperumal, and Wei-ming Su for structure in Fig. 10; Steven Abel, Anupama Amaran, Jennifer Dasta, Tracy Irwin, Jennifer Liu, Sri Namperumal, and Wei-ming Su for technical assistance; Dr. Paul Loach for use of CD spectrometer; Dr. Francis Neuhaus for use of equipment; and Dr. Robert MacDonald for reading the manuscript.

REFERENCES

1. Bennett, V., and Gilligan, D. M. (1993) Annu. Rev. Cell Biol. 9, 27–66
2. Lux, S. E., and Palek, J. (1995) in Disorders of the Red Cell Membrane in Blood: Principles and Practice of Hematology (Handin, R. I., Lux, S. E., and Stossel, T. P., eds) pp. 1701–1818, J. B. Lippincott Co., Philadelphia
3. Gallagher, P. G., and Forget, B. G. (1993) Semin. Hematol. 30, 4–21
4. Davison, M. D., Baron, M. D., Critchley, D. R., and Wootton, J. C. (1989) Int. J. Biol. Macromol. 11, 81–90
5. Koenig, M., Monaco, A. P., and Kunkel, L. M. (1988) Cell 53, 219–228
6. Dubreuil, R. R. (1996) Curr. Top. Membr. 43, 147–167
7. Palek, J., and Sahr, K. E. (1992) Blood 80, 308–330
8. Winograd, E., Hume, D., and Branton, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10788–10791
9. Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C., and Branton, D. (1993) Science 262, 2027–2040
10. Pascual, J., Pfuhl, M., Rivas, G., Pastore, A., and Saraste, M. (1996) FEBS Lett. 383, 201–207
11. Macdonald, R. I., Musacchio, A., Holmgren, R. A., and Saraste, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1299–1303
12. Parry, D. A. D., Dixon, T. W., and Cohen, C. (1992) Biophys. J. 61, 858–867
13. Sambrook, J., Frisech, E. F., andmaniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) Current Protocols in Molecular Biology, Wiley Interscience, New York
15. Wawenius, V.-M., Saraste, M., Salvén, P., Eramaa, M., Holm, L., and Lehto, V.-P. (1989) J. Cell Biol. 108, 79–93
16. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Duberdoeff, J. W. (1990) Methods Enzymol. 185, 60–89
17. Hanahan, D. (1985) in Techniques of Transformation of E. coli in DNA Cloning: A Practical Approach (Glover, D. M., ed.) pp. 109–127, IRL Press at Oxford University Press, Oxford
18. Fasman, G. D. (ed.) (1989) Practical Handbook of Biochemistry and Molecular Biology, pp. 81–82, CRC Press, Inc., Boca Raton, FL
19. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
20. Budzynski, D. M., Benight, A. S., LaBrake, C. C., and Fung, L. W.-M. (1992) Biochemistry 31, 3653–3660
21. Subbarao, N. K., and Macdonald, R. C. (1993) Analyst 118, 913–916
22. Johnson, M. L., and Faunt, L. M. (1992) Methods Enzymol. 210, 16
23. Yang, J. T., Wu, C. C., and Martinez, H. M. (1986) Methods Enzymol. 130, 208–207
24. Pace, C. N., Shirley, B. A., and Thompson, J. A. (1989) in Protein Structure: A Practical Approach (Creighton, T. E., ed) pp. 311–330, IRL Press at Oxford University Press, Oxford
25. Jackson, S. E., Moraci, M., eMasary, N., Johnson, C. M., and Fersht, A. R. (1993) Biochemistry 32, 11259–11269
26. Eftink, M. R. (1994) Biochem. J. 266, 482–501
27. Schols, J. M., Barrick, D., York, E. U., Stewart, J. M., and Baldwin, R. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 185–189
28. Santoro, M. M., and Bolen, D. W. (1993) Biochemistry 31, 4901–4907
29. Yao, M., and Bolen, D. W. (1995) Biochemistry 34, 5771–5781
30. Boyer, C. A., Mann, C. J., and Matthews, C. R. (1993) Protein Sci. 2, 1844–1852
31. Bier, M. (1957) Methods Enzymol. 4, 147–166
32. Laue, W. R., and Contino, P. B. (1992) Methods Enzymol. 210, 448–462
33. Greenfield, N., and Fasman, G. D. (1969) Biochemistry 8, 4108–4116
34. Dill, K. A., and Shortle, D. (1991) Annu. Rev. Biochem. 60, 795–825
35. Ralston, G., Cronin, T. J., and Branton, D. (1990) Biochemistry 29, 5257–5263
36. Bordo, D., and Argos, P. (1991) J. Mol. Biol. 217, 721–729
37. Dubreuil, R. R., Byers, T. J., Sillman, A. L., Bar-Zvi, D., Golstein, L. B. S., and Branton, D. (1989) J. Cell. Biol. 109, 2197–2205
38. Winkelmann, J. C., and Forget, B. G. (1990) Blood 81, 3173–3185
39. Kahana, E., and Gratzer, W. B. (1995) Biochemistry 34, 8110–8114
40. Winder, S. J., Gibson, T. J., and Kendrick-Jones, J. (1995) FEBS Lett. 369, 27–31
41. Benfert, N., Mitchell, T., Lusitini, D., Topouzian, N., and Fung, L. W.-M. (1996) J. Biol. Chem. 271, 30410–30416
42. Deli³va, T. M., Harper, S. L., Koulata, L., Hensley, P., Curtis, P. J., Otvos, L., and Speicher, D. W. (1997) Biochemistry 36, 3991–3997
43. Harris, D. L., and Hudson, B. S. (1990) Biochemistry 29, 5276–5285