Physicochemical and Hydrodynamic Characterization of P-57, a Neurospecific Calmodulin Binding Protein†

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ABSTRACT: P-57 is a neurospecific calmodulin binding protein that was discovered by virtue of its unusual interactions with calmodulin–Sepharose [Andreasen, T. J., Luetje, C. W., Heideman, W., & Storm, D. R. (1983) *Biochemistry 22*, 4615–4618; Cimler, B. M., Andreasen, T. J., Andreasen, K. I., & Storm, D. R. (1985) *J. Biol. Chem. 260*, 10784–10788]. In contrast to other calmodulin binding proteins, P-57 has higher affinity for calmodulin–Sepharose in the absence of calcium compared to that in the presence of calcium. In this study, we report the chemical and physical properties of P-57 purified from detergent-solubilized bovine brain membranes. The amino acid composition of P-57 is distinctive in that it contains a single phenylalanine residue with no other aromatic amino acids and a relatively high percentage of proline and alanine. In the presence of 0.05% Lubrol PX, its predicted secondary structure from circular dichroism spectroscopy is 1% α-helix, 21% β-sheet, and 78% random coil. The hydrodynamic characteristics of the protein–detergent complex and the molecular weight of the protein were determined by gel filtration and sucrose density gradient sedimentation in the presence and absence of calmodulin. The P-57–detergent complex has an apparent Stokes radius (R_s) of 4.58 nm and a sedimentation coefficient ($S_{20,w}$) of 1.44 S while the Stokes radius and $S_{20,w}$ for the P-57–calmodulin–detergent complex are 5.33 nm and 2.32 S, respectively. Perrin analysis of a 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid (AEDANS) derivative of P-57 confirmed the Stokes radius determined by gel filtration. Frictional coefficients of 1.88 and 1.83 for the P-57–detergent and P-57–calmodulin–detergent complexes suggest elongated asymmetric particles. Partial specific volumes of 0.778 and 0.798 mL/g corresponding to molecular weights of 36 800 and 63 213 were obtained for the P-57–detergent and the P-57–calmodulin–detergent complexes, respectively. The estimated molecular weights for P-57 and the P-57–calmodulin complex without detergent are 25 700 and 45 600, respectively, although P-57 runs with an apparent M_r of 54 100 on sodium dodecyl sulfate (SDS)–polyacrylamide gels. These data indicate that P-57 is an elongated calmodulin binding protein with an unusual amino acid composition and atypical behavior on SDS gel electrophoresis.

Intracellular Ca^{2+} acts as a messenger in regulating cellular functions by a variety of mechanisms (Rasmussen & Barrett, 1984). In many instances, the Ca^{2+} binding protein calmodulin (CaM)[1] functions as an intracellular Ca^{2+} receptor and mediates Ca^{2+} stimulation of several enzyme activities [for a review see Manalan and Klee (1984)]. In general, CaM interacts with target enzymes with greater affinity in the presence of Ca^{2+} compared to that in the absence of Ca^{2+} [reviewed in Olwin et al. (1984a)]. For example, the affinity of CaM for the CaM-regulated enzymes, myosin light chain kinase, cyclic nucleotide phosphodiesterase, or phosphorylase kinase is greatly enhanced by Ca^{2+} (Blumenthal & Stull, 1980; Crouch et al., 1981; Burger et al., 1983; Olwin et al., 1984b; Olwin & Storm, 1985).

There are, however, several examples of CaM binding proteins that bind CaM with equal or greater affinity in the absence of Ca^{2+}. Using ^{125}I-CaM and the gel overlay technique, Van Eldik and Burgess (1983) and Burgess et al. (1984) identified several polypeptides from the microsomal fraction of normal and transformed fibroblasts that apparently interacted with CaM with equal affinity in the presence or absence of Ca^{2+}. These peptides had apparent molecular weights of approximately 29 000 and 25 000. P-57 is a protein whose purification from bovine brain membranes was based on its greater affinity for CaM–Sepharose in the absence of Ca^{2+} compared to that in the presence of Ca^{2+}. This protein migrates with an apparent molecular weight of 57 000 on SDS–polyacrylamide gels and photoaffinity cross-links to ^{125}I-labeled azido-CaM with a 1 to 1 molar ratio only in the presence of excess Ca^{2+} chelator (Andreasen et al., 1983). In a subsequent study, P-57 was shown to be widely distributed in various regions of the brain where it accounts for 0.1% of the total protein in white manner and 0.5% of the total protein in cell body rich fractions (Cimler et al., 1985). P-57 was not detected in a variety of other tissues other than retina and spinal cord and is presumed to be a neurospecific protein. In brain, the concentration of P-57 is greater than any other known CaM binding protein, and it has been proposed that it may function to localize CaM at certain sites within the cell and release CaM locally in response to increases in free Ca^{2+}. In this study, we report the physical properties of P-57 including

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[1] Abbreviations: AEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid; CaM, calmodulin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 3-(*N*-morpholino)-propanessulfonic acid; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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its chemical composition, spectral properties, and hydrodynamic characteristics.

Experimental Procedures

Protein Preparations. P-57 was prepared as described by Andreasen et al. (1983) with several modifications. Detergent-solubilized membranes from 1 kg of brain were applied to a 2-L suspension of DEAE-Sephacel equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1% Lubrol PX (buffer A). The protein fraction containing P-57 eluted in a single step from the resin with buffer A containing 150 mM NaCl. This sample was then diluted in half with buffer A; the preparation was adjusted to 2 mM $CaCl_2$ and applied to a 2.5 × 27 cm CaM–Sepharose column. The unbound material was washed from the column as a single peak of protein with buffer A containing 1.1 mM $CaCl_2$. This peak of protein containing P-57 was adjusted to 65% $(NH_4)_2SO_4$ and 2 mM EGTA and allowed to stir for 1 h at 4 °C. The solution was then spun for 1 h at 3000 rpm in a Sorvall RC-3B centrifuge. The float containing P-57 was skimmed off the top, resuspended in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.1% Lubrol PX (buffer B), and dialyzed against 2 × 4 L of buffer B. The dialysate was adjusted to 5 mM EGTA and applied to a second CaM–Sepharose column equilibrated with buffer B. The column was washed with 400 mL of buffer B, and a single peak of protein containing P-57 was eluted with 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 3 mM $CaCl_2$, 1 mM DTT, and 0.05% Lubrol PX.

CaM was prepared as described by Gopalakrishna and Anderson (1982). CaM–Sepharose was prepared from purified CaM and cyanogen bromide activated Sepharose 4B according to the procedure of Westcott et al. (1979).

Amino Acid Analysis. Protein samples were hydrolyzed in sealed tubes in 6 N HCl for 12 h containing a crystal of phenol to protect tyrosine against destruction. Analyses were carried out in a Dionics D-500 amino acid analyzer according to the manufacturer's instructions. The calculated amino acid residues were averaged and rounded to the nearest integer.

Hexosamine Determination. Protein was hydrolyzed in 4 M HCl for 4 h at 100 °C, and the neutralized hydrolysates were analyzed for the presence of hexosamines by gas-phase chromatography using *N*-acetylglucosamine as a standard (Catley et al., 1969).

Determination of Isoelectric Point. The isoelectric point of P-57 was estimated by isoelectric focusing according to O'Farrell (1975) and by the pH-dependent ion-exchange resin binding technique described by Yang and Langer (1985). One-milliliter aliquots of protein were added to cation-exchange resin (QAE-sephadex C-50) which was equilibrated in acetic acid–NaOH buffer in increasing increments from pH 4.0 to pH 7.0. The protein was allowed to equilibrate with the resin for 5-10 min at 4 °C. The resin was pelleted by centrifugation and the supernatant evaluated for protein content. A plot of pH vs. protein concentration was linear, and the 50% point was used as an estimate of the isoelectric point. Bovine serum albumin, which has a p*I* of 5.2, was used as a standard.

Spectroscopy. Ultraviolet absorption spectra were recorded from 350 to 240 nm with a Beckman DU spectrophotometer using 1-cm path length cells. Circular dichroism (CD) spectra were measured between 260 and 210 nm with a Jouan Jobin Yvon Dichrographe III spectropolarimeter using 0.1-cm path length cells. Samples were prepared by extensive dialysis against 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX and subsequent centrifugation at 290000*g* to remove particulate matter. Measurements were made at ambient temperature.

Gel Filtration Analysis. Column chromatography was performed with Sepharose S-200 resin in a 2.5 × 95 cm column. Samples or standards in a maximum volume of 0.5 mL were applied to the column equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX (buffer C). Fractions (1.7 mL) were collected by gravity at a flow rate of 15 mL/h and analyzed for protein content. The following proteins with known Stokes radii were used to calibrate the column: thyroglobulin (8.5 nm), catalase (5.2 nm), bovine serum albumin (3.5 nm), ovalbumin (2.84 nm), and beef heart cytochrome *c* (1.72 nm). The void and total volumes were determined with blue dextran and 5,5′-dithiobis(2-nitrobenzoic acid), respectively. Samples and standards were chromatographed a minimum of 3 times. High-pressure liquid chromatography (HPLC) was performed on an LKB HPLC equipped with a Model 2150 pump, a 2138 Uncord controller, and a tandem of TSK G-3000 (7.5 × 600 mm) gel-filtration columns. Chromatography was performed in 10 mM sodium phosphate, pH 6.0, with 6 M guanidine hydrochloride. Bovine serum albumin (3.5 nm), ovalbumin (2.84 nm), carbonic anhydrase (2.14 nm), and α-lactalbumin (1.65 nm) were used as standards.

Sucrose Density Gradient Centrifugation. Linear sucrose gradients (5–20%) were poured in 13 × 51 mm polyallomer centrifuge tubes. The gradients had a total volume of 3 mL and were layered on top of a 0.4-mL 40% sucrose cushion. Each sucrose solution contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.05% Lubrol PX. Sample volumes were 0.2 mL and contained 0.1 mM phenylmethanesulfonyl fluoride in addition to the buffer described above. In D_2O sucrose gradients, the D_2O replaced 96% of the H_2O. Gradients were centrifuged for 16 h at 48000 rpm at 5 °C in a Beckman SW-65 rotor in a Beckman L5-65 or L8-M centrifuge. Gradients were fractionated and the refractive index was determined on selected samples. The density for each fraction was determined by linear interpolation. The position of migration of samples in the gradient was determined by analysis of fractions run on SDS–polyacrylamide gels. The following proteins with known sedimentation coefficients ($S_{20,w}$) were used as standards: human serum albumin (4.60 S), α-chymotrypsinogen (2.53 S), and beef heart cytochrome *c* (1.71 S).

Hydrodynamic Analysis. Sedimentation coefficients were determined by sedimentation in sucrose density gradients. The partial specific volumes of the protein-detergent complexes were determined by differential sedimentation in sucrose gradients in the presence of H_2O or D_2O (Clarke, 1975). Apparent Stokes radii (R_s) were determined by gel filtration. The partial specific volume of P-57 was determined from the amino acid content as described by Cohn and Edsall (1943), and the partial specific volume of CaM was taken to be 0.712 from Crouch and Klee (1980). Frictional coefficients were calculated as described by Tanford (1961) and the axial ratios determined according to Cantor and Schimmel (1980). By use of a dissociation constant of 36 nM for the P-57–CaM complex (unpublished observations), the hydrodynamic properties of the P-57–CaM complex were determined with mixtures of P-57–CaM at molar ratios that ensured at least 99% saturation of P-57 with CaM.

Preparation of Fluorescent Proteins. The 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid (AE-

DANS) derivative of P-57 was prepared as described by Laporte et al. (1981) with some modifications. The reaction mixture containing 1.25 mg of P-57 in buffer C and 15 mM AEDANS was gently shaken for 24 h at 4 °C. The labeled protein was separated from unreacted components by gel filtration with a Sepharose G-25 column equilibrated in 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 7.2, 150 mM KCl, 0.25 mM EGTA, and 1 mM DTT. The amount of probe incorporated into the protein was determined by assuming a molar extinction coefficient of $E_{337} = 6000\ M^{-1}\ cm^{-1}$ for AEDANS (Hudson & Weber, 1973).

Fluorescence Measurements. Fluorescence measurements were made with an SLM 4800S spectrofluorometer. Samples were prepared in a buffer containing 10 mM Mops, pH 7.4, 1 mM EGTA, 1 mM DTT, and 0.05% Lubrol PX (buffer D), with other additions as described. Sample temperatures were maintained at 25 ± 0.5 °C, and fluorescence anisotropy was determined as previously described (Laporte et al., 1981).

The limiting anisotropy A_0 and apparent Stokes radius R_{app} of AEDANS-P-57 were determined from anisotropy measurements according to a method similar to that of Perrin (1929) and Weber (1953), as described by LaPorte et al. (1981). Fluorescence anisotropy of a sample may be described as a function of solvent viscosity:

$$A^{-1} = A_0^{-1}[1 + (3k\tau/4\pi R_{app}^3)(T/\eta)] \quad (1)$$

where k is the Boltzmann constant, τ is the fluorescence lifetime of the chromophore, T is the absolute temperature, and η is the solvent viscosity. This equation is valid for spheres or randomly labeled particles. In other cases, such as for specifically labeled protein–dye conjugates, the value of R_{app} is sensitive to the orientation of the probe relative to the protein and will not yield the true Stokes radius. Furthermore, for dye–protein conjugates, the value determined for A_0 by linear extrapolation to infinite solvent viscosity is often less than the true limiting anisotropy predicted on the basis of simple rotational diffusion. This discrepancy is attributable to segmental motion within the protein structure and to chromophore motion relative to the bulk of the protein. These motions are typically fast and are reflected in the value of A_0.

Samples consisted of 10 nmol of AEDANS–P-57 in an initial volume of 1.5 mL of buffer D, and T/η was decreased by the incremental addition of buffer C containing 60% sucrose (w/v). A_0 and R_{app} were determined as described under Discussion. The data were then replotted as A_0/A vs. T/η by using eq 1 (Witholt & Brand, 1970). By use of a rearrangement of the Perrin equation, the average rotational relaxation time $\langle p \rangle$ may be calculated:

$$\langle p \rangle = 3\tau A/(A_0 - A) \quad (2)$$

where A is the anisotropy determined prior to the addition of sucrose.

Fluorescence lifetimes were determined by the phase modulation method of Spencer and Weber (1969). Excitation was at the wavelengths indicated, with incident light modulated at 18 MHz. Scattered light was minimized by using a Schott KV 470 filter. To eliminate the influence of Brownian motion on the measured lifetimes, the excitation beam was plane-polarized 35° from the vertical plane (Spencer & Weber, 1970). The sample consisted of 2.8 μM AEDANS–P-57 in buffer D plus 150 mM KCl and was maintained at 25 ± 0.5 °C. Because the lifetimes determined by phase shift and demodulation differed by less than 9%, the average of the two values is reported.

Polyacrylamide Gel Electrophoresis and Protein Determination. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), and gels were silver-stained according to Ansorge (1985). Protein concentration was determined either by amino acid analysis or by Lowry et al. (1952) as modified by Peterson (1983).

Table I: Amino Acid Composition of P-57[a]

amino acid		amino acid	
Ala	52	Met	2
Arg	6	Phe	1
Asp	19	Pro	18
Cys[b]	2	Ser[c]	13
Glu	50	Thr	17
Gly	16	Trp[d]	0
His	3	Tyr	0
Ile	5	Val	5
Leu	2	total	241[e]
Lys	30		

[a] Amino acid composition is based on the average of 24-, 48-, and 96-h acid hydrolysates. [b] Performic acid oxidized prior to acid hydrolysis and calculated from the cysteic acid/alanine ratio. [c] Serine and threonine increased by 10% and 5%, respectively, to compensate for destruction by acid. [d] 48-h alkaline hydrolysis at 135 °C according to the method of Hugli and Moore (1972). [e] Based on a molecular weight of 24900.

Results

Chemical Composition. The amino acid composition of P-57 has several unusual characteristics. The protein contains only one phenylalanine, and it lacks both tyrosine and tryptophan (Table I). Amino acids containing nonpolar side chains account for 42% of the residues, with alanine and proline comprising 22% and 7.5% of the total number of residues. Analysis of the amino acid composition of proteins by the procedure of Bigelow (1967) gives a measure of the average hydrophobicity ($H_{\phi,av}$) of proteins with integral membrane and membrane-associated proteins typically having $H_{\phi,av}$ values of 1197 ± 97 and 986 ± 75 cal/residue (Barrantes, 1975). P-57 has a $H_{\phi,av}$ value of 704 cal/residue, which is comparable to $H_{\phi,av}$ values obtained for a variety of fibrous proteins such as collagens and tropomyosin. Even though P-57 may superficially resemble collagens in a number of its properties, preliminary sequence data distinguishes P-57 from collagens (unpublished observations). The partial specific volume of P-57, calculated from the amino acid composition according to the procedure outlined by Cohn and Edsal (1943), is 0.73 mL/g.

Hexosamine was not detected when P-57 was subjected to acid hydrolysis and subsequent gas-phase chromatography. In addition, P-57 did not bind to a number of lectins including wheat germ agglutinin, indicating that the protein is not a glycoprotein.

Several basic proteins, such as histones and myelin basic protein, have been shown to bind nonspecifically to CaM, which is itself relatively acidic, with an isoelectric point (p*I*) of approximately 4.0 (Grand & Perry, 1980; Itano et al., 1980). The p*I* of P-57 determined by isoelectric focusing or the ion-exchange binding method was 5.2 ± 0.1, making it unlikely that the interaction between P-57 and CaM is due to nonspecific interactions between two oppositely charged proteins. Furthermore, P-57 showed no significant affinity for methionine-oxidized calmodulin, troponin C, or S-100 protein.

Spectroscopic Analysis. P-57 has an unusual ultraviolet absorption spectrum (Figure 1) with two small peaks at 264 and 262 nm. This absorption spectrum is due to the contribution of a single phenylalanine buried under the edge of the peptide carbonyl absorption. The absorption spectrum confirms the amino acid analysis, which reveals the absence of

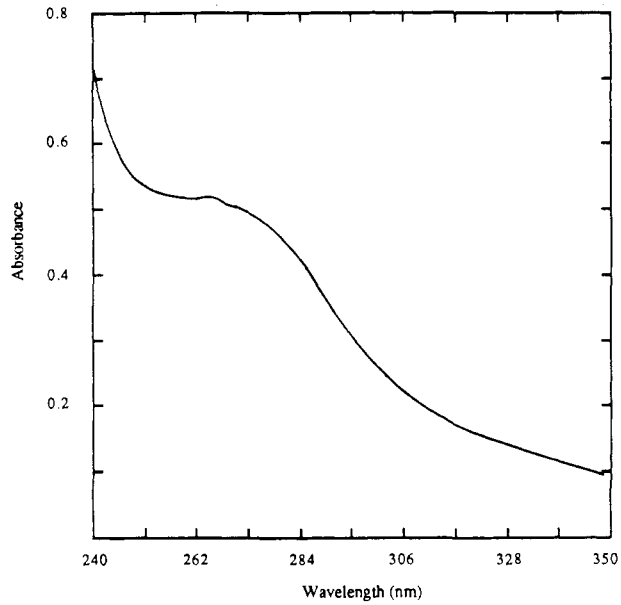

FIGURE 1: Ultraviolet absorption spectrum of P-57. P-57 (63 μM) was analyzed in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX.

Table II: Apparent Molecular Weight of P-57 by SDS–Polyacrylamide Gel Electrophoresis

% polyacrylamide[a]	calcd M_r[b,c]	% polyacrylamide[a]	calcd M_r[b,c]
7.0	53 130	10.0	55 180
8.0	52 310	10.5	55 710
9.0	53 570	11.0	57 150
9.5	53 620	12.0	52,260

[a] Polyacrylamide gels were made from a 30% acrylamide, 2.5% *N,N'*-methylenebis(acrylamide) stock solution. [b] Apparent molecular weights were calculated from a plot of the log M_r vs. migration distance. Phosphorylase B (M_r 96 000), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), and cytochrome *c* (M_r 14,700) were used as molecular weight standards. [c] Average M_r is 54 100 (±610).

the chromophoric residues tyrosine and tryptophan with one phenylalanine present. The molar absorption coefficient for P-57 is E_{264} = 4027.

The CD spectrum of P-57 in the presence of 0.05% Lubrol PX is reported in Figure 2. The negative elipticities at 222, 217, and 208 nm are 621, 634, and 4605 deg·cm^2/dm, respectively. This spectrum was analyzed by the technique of Greenfield and Fasman (1969) to predict secondary structural aspects of P-57. This analysis indicates that P-57 consists of 1% α-helix, 28% β-sheet, and 71% random coil. The secondary structure of proteins with highly ordered structures is accurately predicted from this type of analysis (Greenfield & Fasman, 1969), while those that lack such regularity have larger deviations (Adler et al., 1972). Therefore, the estimate of the percentage of random coil in P-57 is subject to some uncertainty. The relatively large percentage of proline residues obtained from amino acid analysis is consistent, however, with the small percentage of α-helix predicted from analysis of the CD spectrum.

Hydrodynamic Properties. P-57 was so named because the polypeptide ran with an apparent M_r of approximately 57 000 on 10% polyacrylamide–SDS gels (Andreasen et al., 1983). Analysis of the migration of P-57 on a series of different percentage acrylamide gels suggests a somewhat smaller molecular weight of approximately 54 100 (Table II).

The actual molecular weights of P-57 and the P-57–CaM complex were determined by analysis of its Stokes radius and sedimentation coefficients in H_2O and D_2O. The apparent

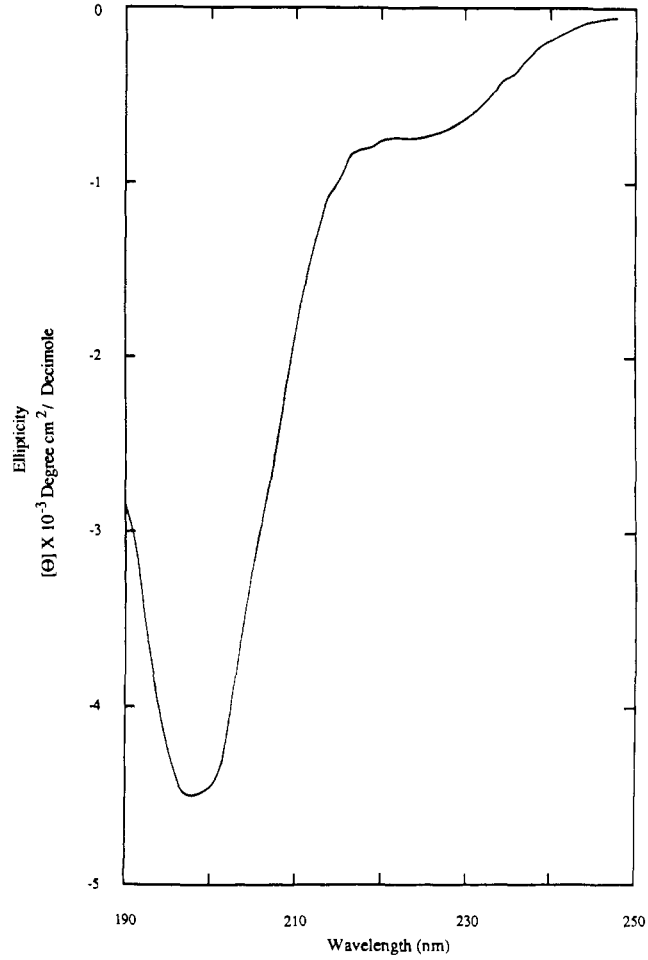

FIGURE 2: Far-ultraviolet CD spectrum of P-57. An 8.1 μM solution of P-57 was analyzed in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX. Negative elipticities at 222, 217, and 208 nm are 621, 634, and 4605 deg·cm^2/dmol, respectively.

Stokes radius (R_s) of P-57 determined by Sepharose S-200 gel filtration was 4.58 nm (Figure 3), and the addition of a 3 to 1 molar excess of CaM to P-57 resulted in a shift of the peak protein fraction to an R_s of 5.33 nm. SDS–polyacrylamide gel electrophoresis of selected fractions revealed P-57 and CaM of equal staining intensities in the peak protein fractions (Figure 3, inset). Andreasen et al. (1983) demonstrated that ^{125}I-labeled azido-CaM and P-57 form a 1 to 1 complex. Therefore, the P-57–CaM complex observed here was assumed to be at a molar ratio of 1 to 1. Unbound CaM migrated with an apparent R_s of 2.68 nm on Sepharose S-200 columns.

P-57 and the P-57–CaM complex were sedimented through 5–20% sucrose gradients in either H_2O or D_2O, and the elution profiles of P-57 and the P-57–CaM complex are reported in Figure 4. In the presence of 0.05% Lubrol PX, P-57–CaM complex migrated with sedimentation coefficients ($S_{20,w}$) of 1.44 and 2.32 S, respectively. The peak of protein corresponding to P-57 sedimented slightly behind cytochrome *c*, while the P-57–CaM complex sedimented slightly slower than α-chymotrypsinogen. A 12.5% polyacrylamide–SDS gel of the peak protein fraction corresponding to the P-57–CaM complex is depicted in the inset of Figure 4. P-57 and the P-57–CaM complex migrated to the same relative position either in the presence or in the absence of proteins used as standards, and there was a slight shift in the position of P-57 and the P-57–CaM complex relative to the standards in gradients run in the presence of D_2O.

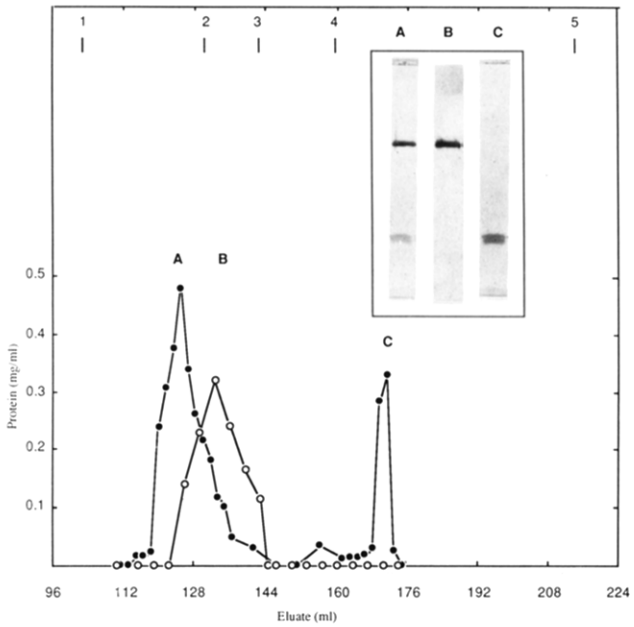

FIGURE 3: Sepharose S-200 chromatography of P-57 and the P-57–CaM complex. P-57 (78 μmol) mixed with 234 μmol of CaM (●) or 78 μmol of P-57 alone (○) was chromatographed on a Sepharose S-200 column equilibrated in 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX. The inset shows a 12.5% polyacrylamide gel of the protein peaks from each chromatogram: (lane A) P-57–CaM complex; (lane B) CaM; (lane C) P-57. Thyroglobulin (1), catalase (2), bovine serum albumin (3), ovalbumin (4), and beef heart cytochrome *c* (5) were used as standards.

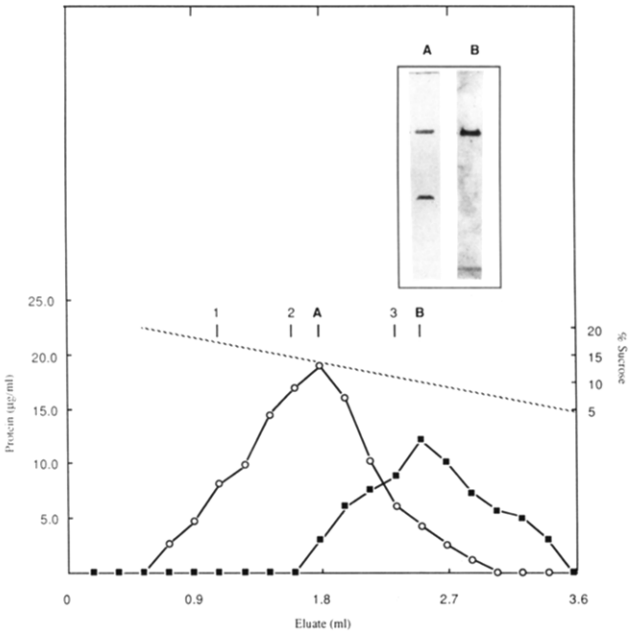

FIGURE 4: Sucrose density centrifugation profiles of the P-57–CaM complex and P-57. P-57 (18.5 μmol) combined with 25 μmol of CaM (○) or 18.5 μmol of P-57 (■) in Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 0.05% Lubrol PX was applied to 5–20% sucrose density gradients as described under Experimental Procedures. The inset shows a 12.5% polyacrylamide gel of the peak protein fractions corresponding to the P-57–CaM complex. Human serum albumin (1), α-chymotrypsinogen (2), and beef heart cytochrome *c* (3) were used as standards.

The hydrodynamic properties of P-57 and the P-57–CaM complex calculated from data obtained from gel filtration and sucrose density gradient centrifugation are summarized in Table III. Substitution of the Stokes radii, sedimentation coefficients, and the partial specific volumes into a modified

Table III: Hydrodynamic Properties of P-57 and P-57–Calmodulin Complex in Detergent

	P-57	P-57–calmodulin
$\bar{v}$ (cm/g), protein[a]	0.730	0.719
$\bar{v}$ (cm/g), protein–detergent[b]	0.796	0.778
$S_{20,w}$ (S), protein–detergent[c]	1.44	2.32
R_s (nm), protein–detergent	4.58	5.33
M_r, protein–detergent[d]	36 800 (±6600)[e]	63 200 (±11 376)
M_r, protein	25 700 (±4600)	47 300 (±8500)

[a] The partial specific volume ($\bar{v}$) for P-57 was determined from the amino acid composition according to Cohn and Edsal (1943). The $\bar{v}$ for CaM was from Crouch and Klee (1980). [b] The $\bar{v}$ for the protein–detergent complex was determined from sucrose gradients in the presence of H_2O and D_2O according to the method of Clarke (1975). [c] Sedimentation coefficients were determined by sucrose density gradient sedimentation. [d] Molecular weights for the protein–detergent complexes were calculated from a modified Svedberg equation: $M_r = S_{20,w}(6\pi)\eta_{20,w}N_{av}R_s/(1 - \bar{v}\rho_{20,w})$, where $\eta_{20,w}$ is the viscosity of water, N_{av} is Avogardro's number, and $\rho_{20,w}$ is the density of H_2O. All results are presented as the mean of at least two separate determinations. [e] Standard error.

Table IV: Predicted Frictional Coefficients and Axial Ratios of P-57 and P-57–CaM Complex in 0.05% Detergent

	f/f_0[a]	axial ratio of prolate ellipsoid[b]	axial ratio of oblate ellipsoid
P-57	1.88	17	24
P-57–calmodulin	1.83	16	23

[a] Frictional coefficients (f/f_0) were calculated by assuming 20% hydration (0.2 g of H_2O/g of protein): $f/f_0 = R_s[4\pi N_{av}/[3M_r(\bar{v} + 0.2/\rho_{20,w})]]^{1/3}$. [b] Axial ratios were determined according to Cantor and Schimmel (1980).

Svedberg equation gave an estimated molecular weight of 36 800 for the P-57–detergent complex and 63 213 for the P-57–CaM–detergent complex. Because of the contribution of detergent to the particle, the calculated partial specific volumes of the protein–detergent complexes are larger than those for the proteins alone. Assuming a partial specific volume of 0.958 mL/g for Lubrol PX (Tanford & Reynolds, 1976) and knowing the partial specific volume of P-57 from its amino acid composition, it was determined that detergent accounts for 29% of the P-57–detergent complex and 25% of the P-57–CaM–detergent complex. The molar ratios of detergent bound to P-57 and P-57–CaM were 18 and 26, respectively. Therefore, the molecular weight for P-57 is 25 700 whereas that for the complex of P-57 and CaM is 47 300. These values are considerably smaller than the apparent molecular weights of 57 000 for P-57 and 70 000 for the P-57–azido–CaM cross-linked complex determined by SDS–polyacrylamide gel electrophoresis in an earlier study (Andreasen et al., 1983).

The frictional coefficients and predicted axial ratios for the P-57–detergent and P-57–CaM–detergent complexes are reported in Table IV. Assuming 20% hydration (0.2 g of H_2O/g of protein), the frictional coefficients of 1.88 for the P-57–detergent complex and 1.83 for th P-57–CaM–detergent complex are larger than those expected for spherical proteins. The axial ratios for these frictional coefficients are reported, assuming either an oblate or a prolate ellipsoid. In either case, it is clear that P-57 is an elongated protein with an axial ratio in excess of 15 and that the complex with CaM also has an unusually large axial ratio. If the degree of hydration is greater than 0.2 g of H_2O/g of protein, then the frictional coefficients would be smaller than those reported in Table IV. Since proteins with a large proportion of random structure tend to be more highly hydrated (Tanford, 1961), the axial ratios presented here should be taken as maximum values. P-57 was

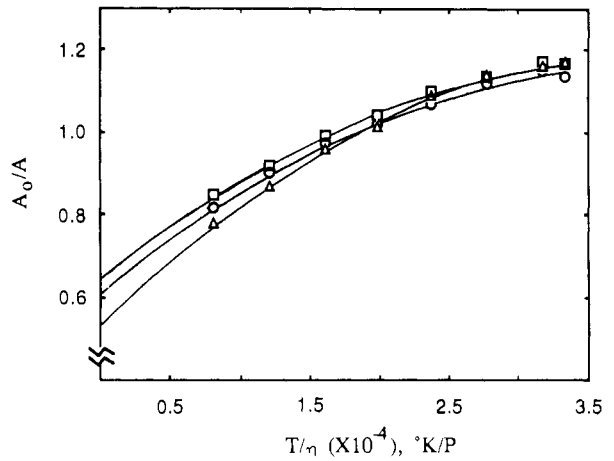

FIGURE 5: Corrected Perrin plots of AEDANS-labeled P-57. Data from uncorrected Perrin plots were processed as described under Results. The sample consisted of 10 nmol of AEDANS–P-57 in 10 mM Mops, pH 7.4, 1 mM EGTA, 1 mM DTT, and 0.05% Lubrol PX. Temperature (T) was maintained constant at 298 K as the sample viscosity (η) was increased by the incremental additions from a 60% sucrose stock in the same buffer. Sample anisotropies were determined after each addition of sucrose. Excitation wavelengths used were 310 (△), 340 (○), and 370 (□) nm, and the excitation resolution was 4 nm. Emitted light was collected through a Schott KV 470 (low-fluorescence) filter.

found to pass through a number of ultrafiltration membranes (Amicon YM 30 and YM 10) that would normally exclude a protein of its size. This observation is consistent with the conclusion that P-57 has an usually high axial ratio and is a relatively long, thin protein.

Denaturation with guanidine hydrochloride altered the structural characteristics of P-57 such that it behaved more like a typical globular protein. In the presence of 6 M guanidine hydrochloride, P-57 migrated as a single peak on a calibrated TSK gel-filtration column (data not shown). The apparent R_s of P-57 under these conditions was 2.28 nm, which would correspond to an M_r of 32 100 ± 3000 for P-57, assuming a direct relationship between Stokes radius and molecular weight in guanidine hydrochloride. This molecular weight is close to the molecular weight of 25 700 ± 4600 described above. These two molecular weights are within the standard errors for these determinations and are consistent with the M_r of 24 900 calculated from the amino acid analysis.

Hydrodynamic Properties of AEDANS–P-57. To further assess the hydrodynamic properties of P-57, fluorescence anisotropy measurements were used to study the rotational diffusion of AEDANS-labeled P-57, as described under Experimental Procedures (Perrin, 1929; Weber, 1953). Fluorescence anisotropy (A) of the protein–dye complex was determined with excitation light at three different wavelengths: 310, 340, and 370 nm. The sample viscosity (η) was varied by the incremental addition of a concentrated sucrose solution, while temperature (T) was held constant at 298 K. Following eq 1, the data for each excitation wavelength were plotted as $1/A$ vs. T/η. This resulted in a nonlinear plot concave toward the latter axis. Because the data could not be accurately modeled by a first-degree polynomial, they were fit to a second-order equation by using a polynomial regression curve fitting program. This technique provided an accurate mathematical model for the observed data ($r > 0.997$). By the use of the coefficients determined from the best-fit polynomial, the first derivative of this expression was taken and used to determine the equation of a line tangent to the polynomial at $T/\eta = 3.343 \times 10^4$ K/P (T/η for the sample prior to addition of sucrose). This tangent line was extrapolated to the ordinate,

Table V: Fluorescence and Hydrodynamic Properties of AEDANS–P-57

parameter	excitation wavelength (nm) 310	340	370
fluorescence lifetime (ns)[a]	12.5 (0.4)[b]	12.9 (0.1)	12.5 (0.4)
anisotropy (3.343×10^4 K/P)[c]	0.0386	0.0409	0.0702
limiting anisotropy[d,e]	0.0453	0.0469	0.0823
$\langle p \rangle$ (ns)[f]	216 (7)	264 (2)	253 (7)
R_{app} (nm)[g]	4.36 (0.01)	4.59 (0.01)	4.34 (0.01)

[a] Determined by the method of Spencer and Weber (1969), as described under Experimental Procedures. [b] Estimated error. [c] Determined by the method of Perrin (1929) and Weber (1953), as described under Experimental Procedures. [d] Anisotropy determined prior to addition of sucrose. [e] Determined by extrapolation of a line tangent to a second-degree polynomial fit of experimental data (see Results). [f] Average rotational relaxation time, calculated by using eq 2. [g] Apparent Stokes radius (R_{app}), calculated by using eq 1.

at which the limiting anisotropy (A_0) could be estimated (Table IV).

After A_0 had been obtained from the extrapolation of a tangent line, the value of A_0 was used to plot the data as a normalized Perrin plot in the form A_0/A vs. T/η (Figure 5). By doing so, the slope of the plot was no longer influenced by A_0, and differences in slopes reflected differences in the apparent Stokes radius (R_{app}) or in the fluorescence lifetime. The normalized Perrin plots also yielded lines concave with respect to the abscissa. These data were fit to a second-degree polynomial. The slope of the line tangent at $T/\eta = (3.343 \times 10^4$ K$)/\eta$ was then set equal to $3\ k\tau/4\pi R_{app}^3$. This allowed the calculation of R_{app}.

The normalized Perrin plots were dependent on excitation wavelength. R_{app} was found to be 4.36, 4.59, and 4.34 nm at 310, 340, and 370 nm respectively, while $\langle p \rangle$ was 216, 264, and 253 ns at these wavelengths (Table V). These determinations of R_{app} were in good agreement with the R_s of 4.58 determined by gel chromatography of unmodified P-57.

Discussion

P-57 is a neurospecific, CaM binding protein that has a greater affinity for CaM in the absence of Ca^{2+} than in its presence, and we have suggested that it may function to localize CaM at specific sites within the cell and release CaM locally in response to increases in free calcium. The binding between P-57 and CaM appears to be specific since there is a Ca^{2+} dependency for the interaction, and oxidized CaM (methionine oxidized) and S-100 protein, as well as troponin C, showed no significant affinity for P-57. Furthermore, P-57 has an isoelectric point of 5.2, which distinguishes it from the family of basic proteins that interact nonspecifically with CaM by virtue of their net positive charge. P-57 may contain, however, a basic region that interacts with CaM in a manner similar to the basic sequence identified and characterized as the putative CaM binding domain from skeletal myosin light chain kinase (Blumenthal et al., 1985; Edelman et al., 1985; Klevit et al., 1985). We have determined some of the properties of P-57 in order to compare it to other CaM binding proteins and to define its distinguishing physical properties. The properties of P-57 reported in this study are different from those of all known CaM binding proteins, and it seems unlikely that P-57 is derived from a larger CaM binding polypeptide through proteolysis since antibodies against the purified protein have failed to recognize larger polypeptides in crude brain extracts prepared under a variety of conditions (Cimler et al., 1985). The compositional and physical properties of P-57 have a number of unique features, which are discussed below.

Since P-57 was purified to homogeneity from Lubrol PX solubilized bovine brain membranes, it was of some interest to determine if its amino acid composition was characteristic of membrane proteins and to determine if the protein binds significant amounts of detergent. Even though there is no generally accepted method for evaluating a protein's hydrophobicity (Eisenberg, 1984), analysis of the amino acid composition of P-57 by the method of Bigelow (1967) or Barrantes (1975) suggests that P-57 does not have an amino acid composition characteristic of integral membrane proteins but is similar to fibrous structural proteins such as collagen or tropomyosin. The hydrodynamic analysis of P-57 in Lubrol PX indicated that 29% of the weight of the particle was detergent, which corresponds to approximately 18 molecules of detergent per mole of P-57. The characterization of the protein–detergent complexes was performed well above the critical micelle concentration for Lubrol PX, which has a micelle molecular weight of 64000 (Tanford & Reynolds, 1976). The relatively low molar ratio of detergent bound to P-57 clearly indicates that the protein was not associated with a detergent micelle and that detergent molecules were probably bound to domains on the molecule, which may be those sites that account for the association of the protein with membranes.

Hydrodynamic data for the P-57–detergent complex calculated from gel filtration and sedimentation in sucrose density gradients indicated an elongated asymmetric shape either in the presence or in the absence of CaM. The axial ratios for the P-57–detergent and P-57–CaM–detergent complexes were 16 and 22, respectively, and the addition of CaM only slightly decreased the axial ratio. The secondary structure of P-57 is characterized by a significant degree of random coil and a low content of α-helix consistent with its high proline content. The apparent Stokes radii determined for AEDANS–P-57 by fluorescence anisotropy at three wavelengths supported the Stokes radius determined for unmodified P-57 by gel filtration. Size determinations by this technique depend upon the behavior of the AEDANS–P-57 complex in solution and upon the degree of randomness of probe incorporation. To assess the degree of randomness, plots of A_0/A vs. T/η as a function of excitation wavelength were constructed (Witholt & Brand, 1970). This approach is based on the assumption that the average angle between vectors representing excitation and emission oscillators will differ with each excitation wavelength used. This angle affects the extent to which various rotational motions of the protein are reflected in the observed rotational diffusion, because the conjugated dye molecules have certain energetically preferred orientations in relation to the protein axes of rotation. The slopes of the normalized Perrin plots vary as a function of excitation wavelength (Weber & Anderson, 1969; Witholt & Brand, 1970). The normalized Perrin plots obtained with AEDANS–P-57 suggested nonrandom labeling of a nonspherical protein.

The apparent molecular weight of 54100 for P-57, determined by SDS–polyacrylamide gel electrophoresis, is substantially greater than the molecular weights of 25700 and 32100 determined for the native protein or the protein in the presence of guanidine hydrochloride, respectively. Although there are several possible sources of error inherent with molecular weights estimated from hydrodynamic measurements, this uncertainty is minimized when the partial specific volume of the protein is known. We place a confidence limit of ±18% on the molecular weight of 25700 obtained from the Stokes radius and sedimentation coefficient. Therefore, it is assumed that P-57 behaves abnormally during SDS–polyacrylamide gel electrophoresis, which could be due to abnormal binding of SDS to the protein or to the maintenance of some secondary structure in the presence of SDS. Proteins that display anomalous behavior by SDS–polyacrylamide gel electrophoresis may have unusual charge such as histones (Panyim & Chalkley, 1971), have an atypical conformation in SDS like collagen (Furthmayr & Timpl, 1971), or be glycosylated such as glycophorin (Grefrath & Reynolds, 1974). P-57 is an acidic molecule with a pI of 5.2, and it is not glycosylated; it has, however, an elongated structure with an axial ratio in excess of 15. Therefore, it seems most likely that the unusual migration of this protein on SDS gels is due to the existence of an atypical conformation in SDS.

In summary, P-57 is a neurospecific, membrane-associated, CaM binding protein of molecular weight 25700 ± 4600 that migrates with an apparent molecular weight of 54100 on SDS gels. P-57 is an elongated molecule with a high percentage of random coil, and it may have hydrophobic domains for detergent binding, although its amino acid composition distinguishes it from integral membrane proteins. Its properties are consistent with the proposal that P-57 is a peripheral membrane associated protein that may bind and localize CaM at the inner surface of the plasma membrane and release CaM in response to increases in free Ca^{2+}.

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Equilibrium and Kinetic Measurements of the Conformational Transition of Thioredoxin in Urea†

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ABSTRACT: Addition of urea to solutions of *Escherichia coli* thioredoxin results in a cooperative unfolding of the protein centered at 6.7 M urea at 25 °C and 5.1 M urea at 2 °C and neutral pH as judged by changes in tryptophan fluorescence emission, far-ultraviolet circular dichroism, and exclusion chromatography. Kinetic profiles of changes in tryptophan fluorescence emission intensity were analyzed following either manual or stopped-flow mixing to initiate unfolding or refolding. Unfolding of the native protein occurs in a single kinetic phase whose time constant is markedly dependent on urea concentration. Refolding of the urea-denatured protein occurs in a multiplicity of kinetic phases whose time constants and fractional amplitudes are also dependent upon urea concentration. Urea gradient gel electrophoretic and exclusion chromatographic measurements suggest the transient accumulation of at least one and likely two compact nativelike intermediate conformations during refolding. Simulations of both electrophoretic and chromatographic results suggest that the intermediate conformations are generated by the concerted action of the middle and fast refolding phases.

The protein thioredoxin obtained from *Escherichia coli* contains a single polypeptide chain of 108 residues of known sequence (Holmgren, 1968). In the native protein, the chain is folded into two domains, a large N-terminal domain having a $\beta\alpha\beta\alpha\beta$ structure and a small C-terminal domain having a $\beta\beta\alpha$ structure (Holmgren et al., 1975). The structure of the native protein unfolds cooperatively and reversibly at neutral pH and 25 °C in solvents containing guanidine hydrochloride in excess of 2 M (Holmgren, 1972; Kelley & Stellwagen, 1984). Unfolding occurs in a single kinetic phase while refolding occurs in three kinetic phases as detected by changes

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