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THE INTERACTION OF CALMODULIN AND POLYLYSINE AS STUDIED BY ^{1}H NMR SPECTROSCOPY AND SEDIMENTATION EQUILIBRIUM CENTRIFUGATION

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SUMMARY. The effects of polylysine on calmodulin were assessed using ^{1}H NMR and sedimentation equilibrium centrifugation. Sedimentation equilibrium centrifugation measurements demonstrated that calmodulin associates with polylysine at calmodulin:polylysine molar ratios ranging from 10:1 to 2.5:1 and when polylysine is increased above the molar ratio of 1:1 a precipitate is formed. At a 1:2.5 calmodulin:polylysine molar ratio, 75% of the calmodulin precipitates from the solution and virtually no polylysine is present in the precipitate. ^{1}H NMR studies of the aromatic region of calmodulin identified chemical shifts of three peaks at a calmodulin:polylysine molar ratio of 1:1. These studies suggest that polylysine associates with calmodulin in aqueous solution and can alter the structure of calmodulin to cause calmodulin self-aggregation.

Calmodulin is a 148 residue acidic calcium-binding protein present in all eukaryotic cells. It activates, in a calcium dependent manner, a large number of enzymes and plays a critical role in a variety of essential cellular functions, including cyclic nucleotide and glycogen metabolism, microtubule assembly and disassembly, smooth muscle contraction, and protein phosphorylation/dephosphorylation [1,2]. These myriad effects are mediated by the binding of calmodulin to a variety of target proteins. Calmodulin has been shown to bind a variety of compounds including: (a) drugs (e.g., trifluoperazine [3] and calmidazolium [4]), (b) peptides (e.g., melittin [5]) and (c) cationic polypeptides, such as histone H2B [6,7], myelin basic protein [7,8], and polylysine [6, 9, 10]. An amphiphilic alpha helical structure containing a cluster of basic residues has been shown to be important for the binding of calmodulin to a series of synthetic basic polypeptides (i.e. polylysine) and calmodulin target proteins [5, 11]. Certain enzymes such as tyrosine kinases, inositol phospholipid kinases and phosphatases are activated by polycations (i.e. polylysine). Specifically, polylysine is essential for the phosphorylation of calmodulin on tyrosine residues by the insulin receptor kinase [12] and enhances serine/threonine phosphorylation

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of calmodulin catalyzed by casein kinase II [13,14]. Polylysine has the ability to promote aggregation of a number of proteins including casein [15], the insulin receptor [16], certain erythrocyte membrane proteins [17], and vesicles composed of biological membranes or of phospholipid/protein mixtures [18]. It has been suggested that *in vitro* polylysine mimics the action of some endogenous intracellular components [15, 19, 20] and that protein aggregation may be an enzyme
regulatory mechanism [15]. Since calmodulin is an important modulator of enzyme activity, we investigated the interaction of polylysine and calmodulin using sedimentation equilibrium and ^1H NMR techniques. These studies provide evidence for polylysine-mediated aggregation of calmodulin.

MATERIALS AND METHODS

Materials. Porcine brain calmodulin was obtained from Ocean Biologics (Edmonds, WA) and made calcium-free by passing over a chelex column (100-200 mesh, sodium form, BioRad, Richmond, CA). Poly-L-lysine (average molecular weights' 3,800 and 44,100), and fluorescamine were obtained from Sigma (St. Louis, MO) and dioxane from Burdick and Jackson (Muskegon, MI). All other reagents were reagent grade quality and purchased from standard sources.

Sedimentation Equilibrium Studies. Samples for both sedimentation equilibrium and ^1H NMR experiments were suspended in 20 mM sodium tetraborate, pH 8.3. For ^1H NMR experiments the buffer was made up in D_2O. These conditions were selected to match the previously published ^1H NMR spectra of calmodulin [21-23], thereby facilitating the identification of specific amino acid residues. Ca^{2+} concentrations in the borate buffer were determined using an Orion Research Ca^{2+} ion electrode 93-20, reference electrode 90-01, and model 601A digital ionalyzer and found to be < 1 μM [24]. A Beckman Airfuge was used to generate sedimentation equilibria for calmodulin/polylysine mixtures. The methodology used followed the general approach of Pollet [25]. Mixtures (in a final volume of 110 μl) of various concentrations of calmodulin and polylysine as indicated in the figure legends and tables were centrifuged at 10±1°C for 24 hours at 50,000 RPM. A micromanipulator-stabilized 24 gauge needle connected to a 100 μl gas-tight syringe was used to obtain 10 μl fractions. Concentrations of calmodulin and polylysine were measured in each fraction and the weight average molecular weight of calmodulin calculated.

The weight average molecular weights (M_W) of calmodulin and calmodulin-polylysine complexes were determined from the graph of the natural log of the protein UV absorbance at 277 nm versus the square of the radial distance of each fraction relative to the solution column meniscus (r_m^2). The slope of this plot was the reduced molecular weight (σ) which was used to calculate M_W for both the protein and the protein-polymer complexes

$$M_W=2\sigma RT/(1-\upsilon\rho)\omega^2,$$

where: R is the molar gas constant, T is the absolute temperature, υ is the partial specific volume, ρ is the solution density, and ω is the angular speed. A constant ρ of 1.009 g/mL was used in all calculations based on previously reported sedimentation equilibrium molecular weight determinations for a variety of proteins at 10°C [26]. The amino acid composition of calmodulin was used to calculate a temperature-corrected [26,27] υ value 0.72 ml/g that was used for all molecular weight calculations.

Calmodulin and Polylysine Concentration Assays. The concentration of calmodulin in sedimentation equilibrium centrifugation fractions was determined using UV absorbance measurements at 277 nm. Since polylysine does not absorb at 277 nm, this assay is specific for calmodulin in each fraction. Each fraction (10 μL) was diluted to 300 μL with borate buffer. The concentration of polylysine in each fraction was determined by a fluorescence assay that measures protein based on the reaction of amines with fluorescamine to produce a fluorescent adduct [28].

The fluorescence contribution of calmodulin was determined from the calmodulin fluorescence calibration curve and subtracted from the total fluorescence of that particular sample. The remaining fluorescence is due to polylysine.

^{1}H NMR Measurements. ^{1}H NMR measurements of 176 μM calmodulin in the presence or absence of varying amounts of polylysine (as indicated in Figures 2 and 3) were performed at 11.7 tesla on a Varian VXR-500 spectrometer operating at a ^{1}H resonance frequency of 499.9 MHz. The spectra were recorded at either 10°C or 25°C using 200 transients, an acquisition time of 0.5 sec., a 70° excitation pulse (5 μsec.) and a 2.5 sec. relaxation delay. Time domain NMR signals were Fourier transformed with exponential apodization resulting in 2 Hz line broadening. Trimethylsilylpropionic acid was added as an internal chemical shift and concentration reference. The residual water signal from the borate buffer solution was suppressed by pre-irradiation at the HOD resonance.

RESULTS

Precipitation of calmodulin by polylysine. A clear solution of calmodulin in D_2O becomes turbid on addition of high molecular weight polylysine (44,100 Da average molecular weight). This occurs even at a very low concentration of polylysine (50,000:1 molar ratio of calmodulin:polylysine). We initially observed this phenomenon indirectly through the disappearance of the aromatic ^{1}H NMR signals of calmodulin after polylysine addition (data not shown). However, utilizing a lower molecular weight polylysine (average molecular weight 3,800 Da) at molar ratios ranging from 10:1 to 2.5:1 calmodulin:polylysine no turbidity is observed. This lower molecular weight polylysine was used in all further studies.

Association of calmodulin and polylysine in solution. Compositional analysis of fractions obtained from calmodulin:polylysine mixtures centrifuged to sedimentation equilibrium were used to characterize the association of these species as shown in Fig. 1. Previous investigations [29] have shown that this analysis, in the case of binding between a non-sedimenting ligand and a protein, produces a slope which corresponds to the number of bound ligands per protein molecule and a y-intercept which is the concentration of unbound (free) ligand. From the data in Fig. 1 a linear regression slope and y-intercept were calculated for each mixture.

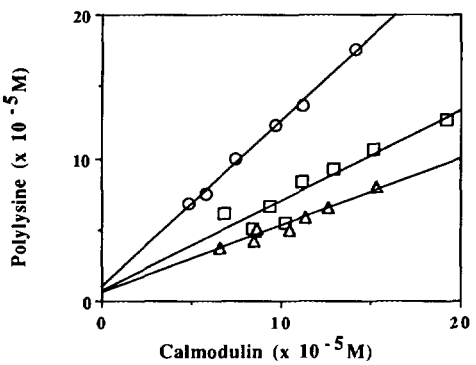

Fig. 1. Polylysine and calmodulin concentrations in sedimentation equilibrium centrifugation fractions using different initial calmodulin:polylysine ratios. The calmodulin:polylysine molar ratios represented by each line are ○ 2.5:1, □ 5:1, and △ 10:1. Each sedimentation equilibrium centrifugation fraction is represented as a single point in each line. The slopes and y intercepts for each line are ○ 0.472, 0.566, □ 0.631, 0.665 and △ 1.162, 0.999, respectively.

The data in Fig. 1 indicate that as the total amount of polylysine (the ligand) increases in the initial mixture (calmodulin:polylysine molar ratios, 10:1 to 2.5:1) the amount of bound polylysine increases. This is demonstrated by the increasing slope (0.472, 0.631 and 1.162) with increasing polylysine concentration in the mixture. Polylysine was centrifuged without calmodulin under the same conditions employed in Fig. 1 as a control for the case of no binding. The polymer alone exhibited virtually no measurable sedimentation under these conditions (data not shown), confirming the presence of a binding interaction when calmodulin is present.

Due to precipitation of mixtures with relative calmodulin:polylysine molar ratios approaching 1:1, insufficient data are available to determine an accurate binding constant or stoichiometry [29]. However, a comparison of the initial and free concentrations of polylysine in the mixtures (Fig. 1) indicates that 80 to 85% of the polylysine is bound by calmodulin over the range studied. If calmodulin is assumed to bind 1 polylysine per molecule, the binding constant (K_a) can be estimated to be on the order of 10^4 M^{-1}.

The M_W of calmodulin in solution is calculated using the same sedimentation equilibrium centrifugation conditions seen in Fig. 1. A steady increase in the M_W is seen as the polylysine content of the mixtures increases (Table 1). These increases are consistent with the data in Fig. 1. It is important to note that the interpretation of M_W data is complicated by anomalously low M_W measured for the protein (11,500 vs the accepted value of 16,700 Da). This phenomenon is well known and has been attributed to polyelectrolyte effects [30], which result from insufficient ionic strength in the buffer. The hypothesis was confirmed by adding 150 mM NaCl to the 2:1 mixture, which increased the calculated M_W from 17,000 to 18,800 (Table 1). Since the 2:1 mixture with added salt presumably represents a measurement free of polyelectrolyte effect, the increase in M_W should reflect the weighted contribution of associated and unassociated calmodulin. As the molecular weight distribution of the 2:1 mixture is not known, interpreting the measured Mw increase in terms of a single protein-polymer complex may not be valid. However, it is interesting that if 85% of the polylysine in the 2:1 mixture associated in a 1:1 complex with calmodulin, the expected Mw for this mixture would be 18,300 which is in close agreement with the measured value of 18,800.

Table 1.
Calculated Weight Average Molecular Weights (M_W) of Calmodulin in Calmodulin:Polylysine Mixtures

Molar Ratio of Calmodulin:Polylysine	M_W of Calmodulin (Da)
Calmodulin (alone)	11,500
10:1	11,100
5:1	11,800
2.5:1	15,400
2:1	17,000
2:1 + 150 mM NaCl	18,800

The weight average molecular weight of calmodulin was determined by sedimentation equilibrium centrifugation described under Materials and Methods at the calmodulin:polylysine ratios indicated. At the highest polylysine concentration (calmodulin:polylysine 2:1), 150 mM NaCl was added to the mix prior to performing sedimentation equilibrium centrifugation.

Table 2.
Composition of Aggregate Formed at 1:2.5 Calmodulin:Polylysine Molar Ratio

Sedimentation-Equilibrium Centrifugation Fraction	Protein Composition	
	Calmodulin μM	Polylysine μM
1	2.735	2.061
2	3.565	2.241
3	3.335	2.349
4	3.618	2.265
5	4.077	2.252
6	5.435	2.709
7	6.882	2.592
8	9.777	3.114
9	13.129	3.663
10	16.988	4.114
Total soluble protein	7.65 nmol	76.316 nmol
Total initial protein	30.59 nmol	76.579 nmol
% soluble protein	25.0%	99.66%

Sedimentation equilibrium studies and measurement of calmodulin and polylysine in each fraction were performed as described under Materials and Methods. The % soluble protein represents the fraction of calmodulin or polylysine in the aqueous phase expressed as a percentage of the initial amount present in the assay.

Composition of the precipitate. At a calmodulin:polylysine molar ratio of 1:2.5 we found that 25% of the calmodulin is in solution while 75% is in the form of a precipitate (Table 2). Interestingly, the calmodulin in this precipitate is not complexed with polylysine which remained in solution. Calmodulin precipitation is inhibited by 150 mM NaCl (data not shown) and 7 mM calcium (Fig. 2) without preventing the calmodulin:polylysine association (*vide supra*).

Effects of polylysine on calmodulin. The interaction between calcium-free calmodulin and polylysine was also studied by ^{1}H NMR spectroscopy. The incremental addition of polylysine to the mixture results in a loss of ^{1}H signal and the development of visible turbidity at molar ratios of 1:1 and 1:2.5 calmodulin:polylysine indicating a loss of calmodulin from solution (Fig. 2). When performed at 25°C, one noticeable change in the aromatic spectrum of calcium-free calmodulin is the emergence of a signal to the high field side of the resonance centered at 6.72 PPM (Fig. 2) as the polylysine concentration increases (for example compare the resonance pattern near 6.7 PPM for calmodulin:polylysine ratios of 1:1 and 5:1). This peak is assigned to protons on Tyr 138 based on previous reports [21-23]. There are two other apparent peak shifts at 7.04 and 7.34 PPM. These peaks are less well defined in the literature but are generally assigned to protons from phenylalanine (Phe) [26]. Since the ^{1}H NMR shifts for the majority of the nine Phe residues in calmodulin overlap significantly, accurate values of aromatic ^{1}H shifts of most Phe residues are difficult to obtain. Other peak changes are due to a decrease in the ^{1}H NMR spectrum signal to noise ratio at the calmodulin:polylysine ratio of 1:1 and 1:2.5 (Fig. 2) which is consistent with calmodulin coming out of solution. The ^{1}H NMR studies were also performed at the same temperature used for the sedimentation equilibrium centrifugation studies, namely 10°C. Changes

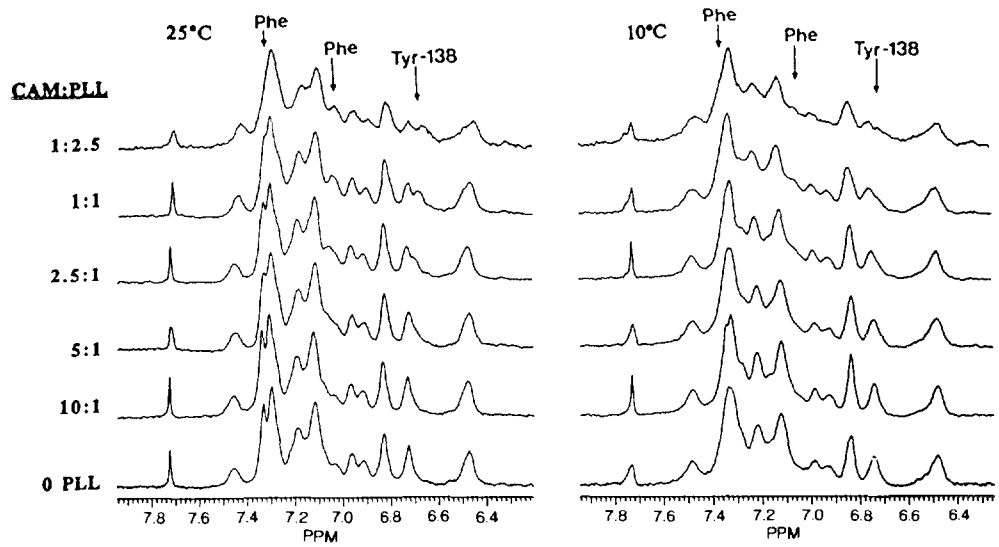

Fig. 2. The aromatic region of the ^1H NMR spectrum of calcium-free calmodulin in the presence of varying polylysine concentrations. Conditions for ^1H NMR spectroscopy of calmodulin are described under the Materials and Methods. The molar ratios of calmodulin (CAM) to polylysine (PLL) are indicated on the left side of the spectrum. 0 PLL corresponds to calmodulin alone. ^1H NMR was carried out at either 25°C (left panel) or 10°C (right panel). Polylysine was added in increments to a single calmodulin sample to achieve the six spectra shown in order of increasing (from bottom to top) polylysine concentrations at a constant calmodulin concentration (176 μM). Representative experiments are shown. The chemical shift assignments for Tyr 138 (6.69 PPM) and Phe (7.04 and 7.34 PPM) are based on previous reports (21-23).

are less apparent at 10°C although the decrease in signal to noise ratio is still apparent (Fig. 2, right panel).

No changes in the aromatic ^1H NMR spectrum are noted when monomeric lysine is added under similar conditions (data not shown). Changes in the aromatic region of the ^1H NMR spectrum of calmodulin occur at the same calmodulin:polylysine ratio that caused aggregation (observed by sedimentation equilibrium). Addition of calcium markedly alters the calmodulin-polylysine interaction. In contrast to calcium-free calmodulin, there are no apparent changes in the aromatic region of the ^1H NMR spectrum of calmodulin at either 25°C or 10°C in the presence of saturating calcium concentrations (Fig. 3).

DISCUSSION

Calmodulin and polylysine associate in aqueous solution. Above a calmodulin:polylysine molar ratio of 1:1, a visible precipitate is formed that contains calmodulin but not polylysine (Table 2). The mechanism whereby polylysine induces calmodulin aggregation is not known. Arguments can be made both to support or refute the precipitation of calmodulin by polylysine as being a "salting out" reaction. ^1H NMR studies indicate that polylysine affects the surface hydrophobicity of calmodulin (Fig.2 and *vide infra*), which has been shown to be related to the salting out behavior of proteins [31,32]. On the other hand, classical salting out reactions are generally accomplished at much higher salt concentrations [32]. Furthermore, we observed that

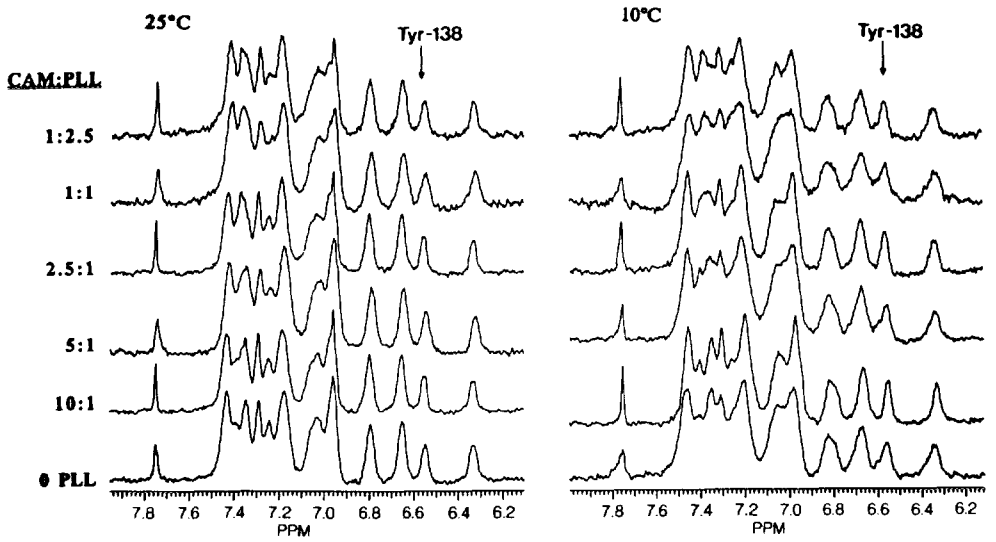

Fig. 3. The aromatic region of the ^1H NMR spectrum of calmodulin in the presence of 7 mM $CaCl_2$ and varying polylysine concentrations. Conditions for ^1H NMR spectroscopy of calmodulin are described under the Materials and Methods. The molar ratios of calmodulin (CAM) to polylysine (PLL) are indicated on the left side of the spectrum. 0 PLL corresponds to calmodulin alone. ^1H NMR was carried out at either 25°C (left panel) or 10°C (right panel). Polylysine was added in increments to a single calmodulin sample to achieve the six spectra shown in order of increasing (from bottom to top) polylysine concentrations at a constant calmodulin concentration (176 μM). Representative experiments are shown. The assignment of Tyr 138 at approximately 6.54 PPM is based on previous reports (21-23).

150 mM NaCl prevents calmodulin precipitation at a calmodulin:polylysine molar ratio of 1:2.5. An alternative hypothesis to explain the polylysine-induced aggregation of calmodulin is related to charge neutralization. Normally the highly acidic nature of calmodulin deters aggregation. However, association with polylysine (a highly basic polypeptide) may neutralize the acidic regions of calmodulin and render the aggregation of calmodulin more energetically favorable. A similar mechanism has been demonstrated for polylysine-induced fusion of acidic phospholipid vesicles, which is maximal when the net charge on the aggregated system is near zero [33]. Irrespective of the mechanism one would expect polylysine to be incorporated into the precipitate, but surprisingly it is not.

Calcium changes the conformation of calmodulin to stabilize its tertiary structure and expose hydrophobic regions [34]. The presence of saturating calcium concentration inhibits the precipitation of calmodulin (Fig. 2) without affecting the association of calmodulin and polylysine. Presumably calcium induced conformational alterations prevent the association of calmodulin molecules.

^1H NMR studies of the aromatic region of calmodulin demonstrate changes in the spectrum that are consistent with conformational alterations resulting from the addition of polylysine. These studies indicate that a residue consistent with Tyr 138 and residues associated with Phe are affected by the binding of polylysine. Although the exact binding site for polylysine is not known, three highly acidic regions in the calmodulin molecule that may interact with the basic residues of polylysine have been suggested by Sacks et al. [10]. One of the potential binding sites is located in

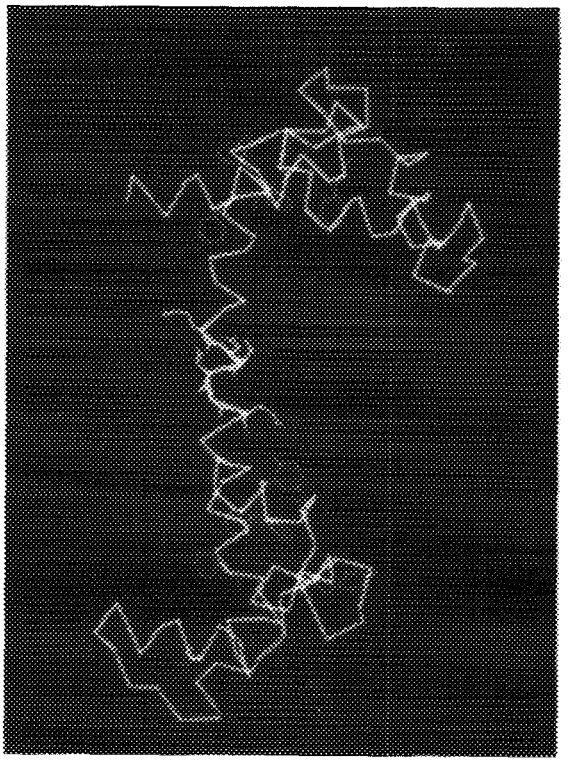

Fig.4. A schematic drawing of the proposed binding site for polylysine in calmodulin. This schematic representation of the three dimensional structure of the calmodulin molecule demonstrates the relative positions of the aromatic residues, shown in red,and the proposed polylysine binding site (amino acid residues 78-87), shown in green. Tyrosine 138 is the aromatic residue closest to the proposed polylysine binding site and is proximal to Phe 89 and Phe 141.

the central helix of calmodulin (amino acids 78-87). If polylysine associates with calmodulin at this site, then Tyr 138 which is the closest aromatic residue is most likely to be affected. Tyr 138 is known to make hydrophobic contacts with two other aromatic residues namely Phe 89 and Phe 141 [35]. This model is illustrated in Fig. 4 and is consistent with the ^{1}H NMR spectra that show Tyr 138 and Phe residues as being shifted by polylysine (Fig. 2).

Our studies are consistent with the ability of polylysine to aggregate proteins [15-18]. In a recent study [15] casein kinase II activation by polylysine was shown to parallel the aggregation of casein and other natural substrates of the enzyme. The nature of the aggregate affected the activity of casein kinase II. Based on these data it was suggested that casein aggregation via endogenous polylysine-like basic proteins could be a mechanism whereby organized casein forms structures in which enzyme is then incorporated, producing a more active conformation. In an analogous manner, endogenous polylysine-like basic proteins may aggregate calmodulin to form organized structures in which calmodulin-dependent enzymes are incorporated and activated.

In summary, we have demonstrated that low molecular weight polylysine forms an association with calmodulin in aqueous solution. This association results in a concentration-dependent conformational alteration of calmodulin that occurs concomitantly with calmodulin

aggregation. Analogous intermolecular interactions of endogenous polylysine-like basic proteins with calmodulin may potentially modify the activity of intracellular calmodulin-dependent enzymes.

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