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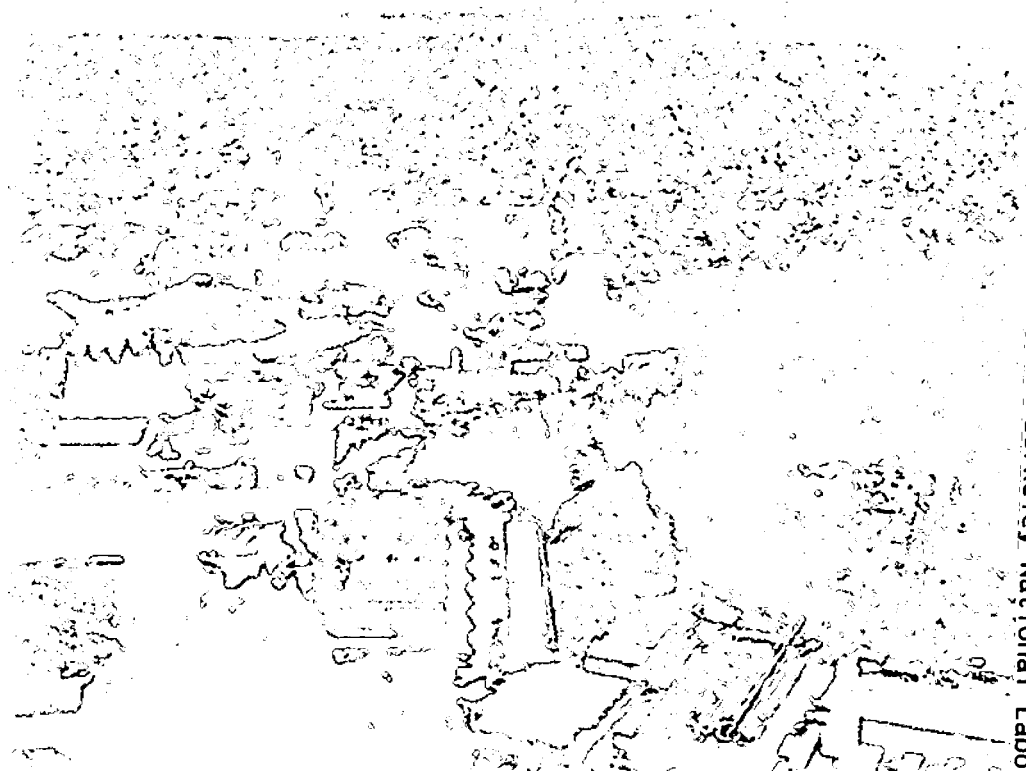
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Chemical Sciences Division

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Interactions of Proteins in Aqueous Electrolyte Solutions from Fluorescence-Anisotropy and Circular-Dichroism Measurements

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Abstract: Understanding aqueous protein-protein interactions is crucial for development of a molecular-thermodynamic model for salt-induced protein precipitation, and for determination of optimal conditions for this and other protein-separation processes. In addition, protein interactions are important in many disease states, including cataracts formation and β -amyloid diseases. Fluorescence anisotropy provides a means to measure intermolecular interactions. In this work, monomer-dimer equilibrium of the peptide T4 LYS(11-36) was studied by fluorescence anisotropy over the pH range 4 to 7 and the NaCl concentration range 0.0 to 1.0 M, in 25-mM sodium phosphate buffer. This 26 amino-acid peptide is derived from the β -sheet region of the T4 lysozyme molecule and has the potential to form amyloid fibrils. The association constant for dimerization increases with rising pH and ionic strength. The potential of mean force for peptide-peptide interactions was calculated from these association constants. Circular dichroism

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measurements show that the peptide becomes more structured as the pH rises, possibly contributing to increased association.

Introduction

A quantitative understanding of aqueous protein-protein interactions has several applications. One important application is to establish a molecular-based theory for salt-induced protein precipitation, a common first-step method for protein recovery. At present, most salting-out procedures are based on experience, trial-and-error methods and empirical correlations. Consequently, selection of a good separation procedure may be labor-intensive and costly. Through measurements of protein-protein interactions under dilute-solution conditions, a model can be developed for predicting phase separation of proteins in concentrated electrolyte solutions.

Protein-protein interactions are also important in certain disease states. In Alzheimer's disease, for example, it has been hypothesized that monomers or dimers of the A β protein associate to form protofibrils with β -sheet structure [Walsh et al. (1997), Harper et al. (1997), Shen and Murphy (1995)]. These protofibrils lengthen to full-length fibrils that form clusters. In time, the plaques which accompany the disease are observed. There are other diseases that involve protein aggregation and deposition, including cataract formation, Huntington's disease and bovine spongiform encephalopathy (Mad Cow disease). Determining the intermolecular forces that lead to protein aggregation can thus play a role in understanding how these diseases arise.

This work is concerned with protein-protein interactions that may be related to β -amyloid formation. Various forms of lysozyme are capable of undergoing amyloid fibrillogenesis. Human lysozyme variants have been shown to form fibrils with β -

structure [Booth et al. (1997)]. In addition, peptide fragments from the β -sheet region of hen egg-white lysozyme associate to form extensive β -structure [Yang et al. (1994)]. In this work, the interactions that lead to β -amyloid fibrillogenesis are examined using fluorescence anisotropy measurements of the peptide T4 LYS(11-36), a 26 amino-acid peptide derived from the β -sheet region of T4 lysozyme. Circular dichroism spectropolarimetry is used to determine whether secondary structure of the T4 lysozyme peptide plays a role in association. The two-body interactions between peptide molecules are modeled using a potential of mean force composed of a repulsive hard-sphere potential, an electric double-layer-repulsion potential, an attractive dispersion potential and an attractive site-specific square-well potential.

Fluorescence Anisotropy and Brownian Motion

Changes in the association state of molecules are reflected by changes in molecular motion. Fluorescence anisotropy measurements are useful for investigating these changes provided that fluorescently labeled molecules are available. The equation governing rotational Brownian motion is [Einstein (1906), Bentley et al. (1985), Weber (1953)]:

$$D_r = \frac{RT}{6\eta V} \quad (1)$$

where D_r is the molecular rotational diffusion coefficient, R is the universal gas constant, T is the absolute temperature, η is the solution viscosity and V is the molar volume, given by [Lundblad et al. (1996), Lakowicz (1983)]:

$$V = M(\bar{v} + h) \quad (2)$$

Here, M is the molecular weight of the molecule, \bar{v} is the partial specific volume and h is the hydration. As the solution temperature increases, the molecules tumble at an increasing rate; as the solution viscosity or the particle volume increase, the molecules rotate more slowly. Association of molecules results in an increase in particle volume.

While equation 1 fully describes rotational diffusion, it cannot be used directly for analysis of fluorescence-anisotropy data. Instead, we use the Perrin equation that relates anisotropy to molecular motion [Perrin (1926)]:

$$A = \frac{A_0}{1 + (RT\tau/\eta V)} \quad (3)$$

Here, A_0 is a constant representing the anisotropy in the absence of molecular motion and τ is the fluorescence lifetime of the fluorophore used. In this work, the effect of peptide concentration on the measured anisotropy is used to determine the association state of the peptides.

Measurement of Fluorescence Anisotropy

Fluorescence anisotropy is measured by first exciting a molecule with vertically polarized light, then observing the fluorescence intensity of emitted light passing through a polarizer held alternately parallel to and perpendicular to the direction of polarization of the incident light [Lakowicz (1983), Bentley et al. (1985)]. The anisotropy is related to these intensities by [Perrin (1926)]:

$$A = \frac{I_{//} - I_{\perp}}{I_{//} + 2I_{\perp}} \quad (4)$$

where I_{\parallel} is the intensity of emitted light measured in the direction parallel to excitation and I_{\perp} is the intensity of emitted light measured in the direction perpendicular to excitation.

Relation of Anisotropy to Association Constant, K_a

Anisotropy is measured as a function of peptide concentration to provide information on monomer-dimer equilibrium. The sample solutions contain both fluorescently labeled and unlabeled peptide molecules. Thus, reversible binding of monomers leads to three types of dimer molecules:



where L is the unlabeled T4 lysozyme peptide (11-36) and L^* is the labeled peptide. (The peptide is labeled with 7-methoxycoumarin-3-carboxylic acid.) Because the mole ratio of labeled to unlabeled peptide is approximately 1:9, the concentration of the dimer L_2^* is small compared to the concentration of the other dimers, and can therefore be neglected. At equilibrium, the remaining association constants in equation (5) are given by:

$$\begin{aligned}
 K_{L_2} &= \frac{[L_2]}{[L]^2} \\
 K_{L^*L} &= \frac{[L^*L]}{[L^*] \cdot [L]}
 \end{aligned} \tag{6}$$

Assuming that the fluorescent label does not affect the interactions between the peptide molecules, the two association constants in equation (6) are equal and equivalent to K_a .

For a mixture of monomers and dimers carrying the same fluorophore [Weber (1952)]:

$$\begin{aligned} A &= A_f F_f + A_b F_b \\ F_f + F_b &= 1 \end{aligned} \quad (7)$$

where the subscript f denotes molecules in the monomer (free) form and b denotes molecules in the dimer (bound) form. A_f is the infinite-dilution anisotropy, while A_b is the limiting value at very high peptide concentrations where all molecules are in the bound form. F_b , the fraction of labeled molecules in the dimer form, is given by:

$$F_b = \frac{[L^* L]}{[L^*]_T} \quad (8)$$

where $[L^*]_T$ is the total concentration of labeled peptide in the mixture.

The mass-balance equations for this system are:

$$[L^*]_T = [L^*] + [L^* L] \quad (9)$$

$$[L]_T = [L] + [L^* L] + 2[L_2] \quad (10)$$

$$[L^*]_T = \lambda^* [\Lambda]_T \quad (11)$$

$$[L]_T = \lambda [\Lambda]_T \quad (12)$$

where $[L]_T$ is the total unlabeled peptide concentration, $[\Lambda]_T = [L^*]_T + [L]_T$ is the total molar peptide concentration, λ^* is the mole fraction of labeled peptide, and λ is the mole fraction of unlabeled peptide. Combination of equations (6)-(12) relates the experimental anisotropy to the association constant:

$$[\Lambda]_T = \frac{\left[\left(\frac{A - A_f}{A_b - A_f} \right) + \left(\frac{A - A_f}{A_b - A_f} \right)^2 \right]}{K_a \cdot \left[1 - \left(\frac{A - A_f}{A_b - A_f} \right) \right]^2 \cdot \left[\lambda - \left(\frac{A - A_f}{A_b - A_f} \right) \lambda^* \right]} \quad (13)$$

$[L^*]_T$ and $[L]_T$ are measured experimentally to obtain λ^* , λ , and $[\Lambda]_T$. Parameters A_f and A_b are estimated by plotting the experimental anisotropy, A , versus $\log [\Lambda]_T$ and extrapolating (see Figure 1). Nonlinear least-squares regression (using the program SigmaPlot) is used to fit the data to equation (13) and to obtain the association constant for dimerization, K_a .

Calculation of the Potential of Mean Force

The association constant can be related to the sum of several contributions to the potential of mean force. The association in this system is assumed to be driven primarily by specific attractive interactions between the peptide molecules [Fink (1998), Havel et al. (1986)]. These attractive interactions arise due to hydrophobic forces, hydrogen-bond formation, and short-range attractive electrostatic forces [Curtis et al. (1998)]. The potential of mean force also depends on the size and net charge of the peptide molecules. Thus, the potential of mean force is given by a sum of pairwise potentials:

$$W(r) = W_{hs}(r) + W_{elec}(r) + W_{disp}(r) + W_{spec}(r) \quad (14)$$

where r is the center-to-center distance between peptide molecules. Here, $W_{hs}(r)$ is the peptide hard-sphere potential, $W_{elec}(r)$ is the electric double-layer-repulsion potential, and $W_{spec}(r)$ is a square-well potential that accounts for specific interactions between two

peptide molecules. Derjaguin-Landau-Verwey-Overbeek (DLVO) theory accounts for the first three terms in the sum [Verwey and Overbeek (1948)].

The hard-sphere potential is given by [Verwey and Overbeek (1948)]:

$$W_{hs}(r) = \begin{cases} \infty & \text{for } r \leq \sigma \\ 0 & \text{for } r > \sigma \end{cases} \quad (15)$$

where σ is the effective spherical diameter of the peptide. The electric double-layer-repulsion potential is derived from Debye-Hückel theory [Verwey and Overbeek (1948)]:

$$W_{elec}(r) = \frac{z^2 e^2 \exp[-\kappa(r - \sigma)]}{4\pi r \epsilon_0 \epsilon_r (1 + \kappa\sigma/2)^2} \quad \text{for } r > \sigma \quad (16)$$

where z is the valence of the peptide, e is the unit charge on an electron, $4\pi\epsilon_0$ is the dielectric permittivity of free space, and ϵ_r is the relative dielectric permittivity of water; κ is the inverse Debye length given by:

$$\kappa^2 = \frac{2e^2 N_A I}{kT \epsilon_0 \epsilon_r} \quad (17)$$

where N_A is Avogadro's number and I is the salt ionic strength. When the ionic strength of the salt is below 0.1 M, it is reasonable to approximate the salt as a point charge. As ionic strength increases, the Debye-Hückel model breaks down because ionic size becomes significant. Fortunately, however, the effect of ion size in $W_{elec}(r)$ is of minor importance because at high salt concentrations, the electric double-layer repulsion tends to vanish due to screening.

The attractive dispersion potential for these molecules is well approximated by [Nir (1976)]:

$$W_{disp}(r) = -\frac{H}{36} \left(\frac{\sigma}{r} \right)^6 \quad (18)$$

where H is the effective Hamaker constant for the peptide-peptide interaction. The Hamaker constant is primarily a function of the chemical composition of the peptide [Nir (1976)]. The ionic strength has little effect on the dispersion interaction because the time constant for spatial adjustment of ions in the double layer is much larger than that for electronic fluctuations between atoms [Israelachvili (1992)]. The Hamaker constant of a protein is on the order of $5kT$ [Nir (1976)].

The site-specific square-well potential developed by Wertheim [Wertheim (1986)] represents binding between two attractive sites; each site is located on the surface of a distinct peptide molecule. The orientation-dependent potential is given by:

$$\phi(r) = \begin{cases} -\zeta & \text{for } x < x_c \\ 0 & \text{for } x > x_c \end{cases} \quad (19)$$

where x is the distance between the sites and x_c is the cutoff distance of the attractive potential. The function $\phi(r)$ is orientation-averaged to obtain the spherically symmetric potential $W_{spec}(r)$. To ensure that only dimers are formed, the cutoff distance for sites located on the surfaces of the molecules must be less than or equal to 0.134σ [Wertheim (1986)]. Since we assume only a monomer-dimer association, this cutoff distance is used in the present work. The square-well depth, ζ , is a model parameter.

The association constant, K_a , is related to the potential of mean force by [Chandler (1987)]:

$$K_a = \frac{1}{2} \int_0^\infty S(r) \cdot \exp[-W(r)/kT] \cdot 4\pi r^2 dr \quad (20)$$

where $S(r)$ is defined as:

$$S(r) = \begin{cases} 1 & \text{for } r < r_c \\ 0 & \text{for } r \geq r_c \end{cases} \quad (21)$$

and $r_c = x_c + \sigma$. Values of ζ are calculated from experimental K_a .

Results

Anisotropy Measurements. (Experimental details are given in the Appendix.)

The anisotropy of T4 LYS(11-36) was measured in 25 mM sodium phosphate buffer at pH 4, 5, 6 and 7; results are shown in Figure 2. The total peptide concentration, $[\Lambda]_T$, was varied in the range 0.5 to 160 μM . All measurements were performed at 18°C. Anisotropy increases with rising peptide concentration, signifying greater binding of peptides. At pH 4, anisotropy increases slightly as concentration rises, indicating that little binding occurs at this low pH. As pH rises, the anisotropy becomes a stronger function of concentration, indicating increased binding, as expected, because the isoelectric point of the peptide is pH 9.9.

Figures 3 and 4 show the ionic-strength dependence of the anisotropy at pH 4 and 5. At pH 4, increasing the ionic strength slightly to 0.1 M has little effect on the concentration dependence of anisotropy. As ionic strength increases further, a significant concentration dependence is observed. Similar behavior is observed at pH 5.

Association Constants. Association constants for dimerization of T4 LYS(11-36) are calculated by fitting the experimental data with equation (13). For these measurements, $\lambda=0.9$ and $\lambda^*=0.1$. Parameters A_f and A_b are estimated by plotting the experimental anisotropy, A , versus $\log [\Lambda]_T$ and extrapolating (see Figure 1). A_f and A_b are 0.085 and 0.3, respectively; they are assumed independent of pH and salt concentration. The best-fit curves are shown in Figures 2 through 4. Figure 5 shows the

association constant for peptide dimerization as a function of pH. The association constant increases with pH. The association constants shown in Figure 6 are for measurements at pH 4 and 5 with sodium-chloride concentrations in the range 0.0 to 1.0 M. The association constant increases with salt concentration.

Potential of Mean Force. The potential of mean force is calculated using equations (14)-(21). The peptide diameter is 18.8 Å (see Appendix). The peptide charge, shown in Table 1, was calculated using results from free-amino-acid titration in 0.1 M KCl. The Hamaker constant is $5kT$. Two sets of calculations are shown in Figures 7 and 8; in one set, the DLVO potentials and the specific-interaction potential are used and in the other, only the hard-sphere and specific-interaction potentials are included. As pH rises, well depth ζ increases for both calculations (see Figure 7). Figure 8 shows that ζ increases with sodium-chloride concentration for both models.

Circular Dichroism (CD) Spectropolarimetry. CD spectra were obtained as a function of pH at 25°C with peptide concentrations in the range 5 to 10 μ M. Because the buffer contribution to the molar ellipticity was significant at 25-mM sodium phosphate buffer, these measurements were taken in 5-mM buffer. Results are shown in Figure 9. The CD spectrum at pH 4 has a minimum at 197 nm, consistent with the peptide having a random coil conformation [Woody (1996)]. This spectrum is similar to that obtained by Najbar and coworkers [Najbar et al. (1997)] at pH 4.8 in 10-mM potassium phosphate buffer. As pH increases to 7, the spectrum develops a minimum at 216 nm, indicating that the peptide adopts a β -strand conformation [Woody (1996)].

Discussion

Strength of Interaction. Interactions between the peptide molecules are driven by attractive forces similar to those observed for various proteins known to undergo amyloid fibrillogenesis. The observed well depths of the specific interaction potential, ζ , are in the range 14-20 kT. At 18°C, this corresponds to bond energies on the order of 34-48 kJ/mol. The strength of a hydrogen bond is approximately 13-30 kJ/mol, while a covalent C-C bond has an dissociation energy of 347 kJ/mol [Stryer (1988)]. The strength of an intramolecular salt bridge can range from zero if it is on the surface, to 21 kJ/mol if it is buried [Creighton, p.293 (1993)]. For a hydrophobic molecule with a diameter of 18.8 Å, the hydrophobic free energy of dimerization is -38 kJ/mol [Israelachvili, p. 132 (1991)].

It is possible that the attractive interaction is due to the formation of a hydrogen bond or to several salt bridges. There are a number of amino acids in the peptide that could participate in such interactions (see Appendix). Alternatively, a hydrophobic bond could be responsible for the strong attraction. There are 10 amino acids in the sequence that can readily participate in hydrophobic interactions.

pH Dependence of Interaction. Increased association is observed with rising pH. The well depth is a stronger function of pH than of ionic strength. Figure 7 shows that there is an appreciable difference in the ζ values obtained when the electric double layer is included compared to when it is neglected. At pH 4, where the peptide molecules have the highest charge, there is a large discrepancy between the two models. At pH 7, where the molecules have the smallest net charge, there is less difference, indicating that it is necessary to include the electric double-layer-repulsion potential in the model. Inclusion

of this potential does not greatly affect the strong pH dependence of the well depth. Thus, there must be some other intermolecular forces that induce the observed pH-dependent interaction. Short-range electrostatic forces may be responsible, in the form of multiple salt bridges. Alternately, hydrogen bonds could cause this strong attraction. It may be that the interaction is not affected by the net charge of the molecule, but rather by the charges on the amino acids that are responsible for binding.

Role of Structure in Association. An increase in secondary structure is another consequence of increasing pH. The formation of β -structure may drive the interaction, suggesting hydrogen-bond formation as the cause of attraction between peptide molecules.

Ionic-Strength Dependence of Interaction. Association also increases with sodium chloride concentration. Figure 8 shows that at low ionic strength there is a difference in the ζ values obtained when the electric double layer is taken into account and when it is not. As the ionic strength increases to 1.0 M NaCl, the differences between the two models declines. There is a larger difference between the models at pH 4 than at pH 5. The difference between the models indicates that screening of the overall charge occurs as the ionic strength increases.

If association in this system is due to short-range electrostatic interactions, these should be screened out as the ionic strength increases, causing a decrease in attraction rather than the observed increase. The ionic-strength dependence of association may be due to the effect of salt on hydrophobic interactions. The effect of salt type on the surface tension of water follows the lyotropic series [Melander and Horvath (1977)]. If the specific interactions in this system are due to interactions between hydrophobic

patches, an increase in the surface tension of water would lead to a decrease in protein surface-water contacts, or, in effect, stronger peptide-peptide attraction. Na⁺ and Cl⁻ ions are intermediate in the lyotropic series, indicating that neither has a strong tendency to increase the surface tension of water.

Conclusions

This work describes interactions of the peptide T4 LYS(11-36) measured by fluorescence anisotropy. The association constants increase with pH and sodium chloride concentration. The observed dimerization equilibrium constants can be described by a potential of mean force consisting of the DLVO potentials and a specific interaction potential. It was shown that specific interactions play a large role in the interactions of the peptide T4 LYS(11-36).

APPENDIX

Experimental Procedures

Peptide Synthesis. T4 LYS(11-36), which has the amino acid sequence EGLRLKIYKDTEGYTIGIGHLLTKS, was assembled on an Applied Biosystems 431A synthesizer using the Fmoc (9-fluorenylmethoxycarbonyl) group for protection of the α -amino function (Dr. David King at the Howard Hughes Medical Institute Peptide/Mass Spectrometry Laboratory). The peptide was cleaved from the resin and deprotected with reagent K. The crude product was purified by reversed-phase, high-performance liquid chromatography using an acetonitrile-water-TFA (trifluoroacetic acid) mixture. Approximately 1 mole TFA per positive charge and 20% H₂O were present in the lyophilized peptide powder. Electrospray ionization mass spectrometry

yielded a molecular weight of 2969.4 Da, consistent with the theoretical value. The peptide has an isoelectric point of 9.9.

Conjugation of Fluorescent Label. 7-Methoxycoumarin-3-carboxylic acid (Molecular Probes, Inc., Eugene, Oregon) was attached to the N-terminus of the protected peptide on the resin by activation with HBTU/HOBt (hydroxybenzotriazol-1-yl tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole). Electrospray ionization mass spectrometry yielded a molecular weight of 3171.7 Da.

Sample Preparation. Peptide solutions for fluorescence polarization measurements were prepared such that the mole ratio of fluorescently labeled to unlabeled peptide was 1:9. This ratio was optimal for ensuring that there was sufficient fluorescent signal for measurement, while at the same time ensuring that the fluorophore concentration was below the level where fluorescence transfer between fluorophores would occur. The solubility of the peptide was greatest at pH 4 in 25-mM sodium phosphate buffer. Therefore, 1-ml peptide solutions with a concentration of 160 μ M were made in 25-mM sodium-phosphate buffer at pH 4 and dialyzed overnight against 1 L of the buffer or salt solution of interest. Dialysis also served to dilute the trifluoroacetic acid (TFA) present in the lyophilized peptide by 1000-fold. This was advantageous, as TFA drastically changes the pH of peptide solutions.

After dialysis, the pH was adjusted to the correct value using either 10 to 20 μ l of 8.5×10^{-5} g/g phosphoric acid or 10 to 20 μ l of 0.5 M NaOH. The 160 μ M peptide solution was diluted with buffer to obtain solutions in the concentration range 0.5 to 160 μ M. The solutions were allowed to equilibrate for two hours before the anisotropy was measured.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy was measured on a SLM 4800S Spectrofluorimeter using the L-format [Lakowicz (1983)]. Excitation was measured at 360 nm and emission was measured at 404 nm, based on excitation and emission spectra obtained for the coumarin-labeled peptide.

Correction of Anisotropy Values. To eliminate background fluorescence, the intensities of the horizontally and vertically polarized components of the buffer were measured and subtracted from the intensities of the peptide solutions before the anisotropy was calculated.

The viscosity of the buffer solution increased slightly due to increasing salt concentration. To measure aggregation, only the effect of changes in molar volume on the anisotropy are of interest. Therefore, the anisotropy was corrected using the viscosity of pure water, η_o , and the viscosity of the salt solution, η :

$$A_{corrected} = A_{observed} \cdot \frac{(1 + RT\tau/\eta V)}{(1 + RT\tau/\eta_o V)} \quad (A.1)$$

Values for η and η_o were obtained from the CRC Handbook of Chemistry and Physics [1986]. The fluorescence lifetime used in the calculation was $\tau=3.01$ ns (personal communication, Molecular Probes, Inc.) The molar volume was calculated using a number-average molecular weight, because the peptide solutions contain both labeled and unlabeled peptide. The partial specific volume, \bar{v} , was estimated to be 0.7 ml/g, based on values for a variety of proteins [Creighton, p.266 (1993)]. The hydration, $h=0.2$ g H₂O/g peptide, obtained from Lakowicz [1983], was converted to ml/g using the appropriate solution density.

Circular Dichroism (CD) Spectropolarimetry. It was not possible to obtain spectra under the solution conditions used for fluorescence polarization measurements because of the large signal of the sodium chloride and the 25-mM sodium phosphate buffer. Thus, unlabeled peptide solutions were prepared in 5-mM sodium phosphate buffer. Peptide concentrations were between 5 and 10 μM . CD spectra were recorded at 25°C on an Aviv Model 62DS Circular Dichroism Spectropolarimeter using a 1-cm pathlength cuvette. The wavelength range was 190 to 250 nm, with intervals of 0.5 nm and a bandwidth of 1.5 nm. Mean molar ellipticities (degree-cm²/decimole-residue), are shown in Figure 9.

Peptide Concentration Measurements. Concentrations were measured using a Beckman DU640 Spectrophotometer. The total molar concentration of labeled peptide, $[L^*]_T$, was derived from the absorbance at 360 nm (the wavelength of maximum absorbance of the fluorophore) and the fact that only one fluorophore was conjugated to a peptide molecule:

$$[L^*]_T = \frac{A_{360}}{\epsilon_C l} \quad (\text{A.2})$$

where A_{360} is the absorbance measured at a wavelength of 360 nm and ϵ_C is the extinction coefficient of the coumarin label; $\epsilon_C = 16,000 \text{ M}^{-1}\text{cm}^{-1}$ (personal communication, Molecular Probes, Inc.). The pathlength of the cuvette, l , is one centimeter. The total peptide concentration was calculated by:

$$[\Lambda]_T = [L]_T + [L^*]_T = \frac{A_{280} - 0.16A_{360}}{\epsilon_L l} \quad (\text{A.3})$$

where A_{280} is the absorbance measured at 280 nm. The extinction coefficient of the peptide, ϵ_L , was estimated from the absorbance of aromatic groups in 0.02-M phosphate buffer at pH 6.5 and 6.0 M guanidinium hydrochloride [Swiss-Protein Database]. The peptide contains three tyrosine groups, thus $\epsilon_L = 3840 \text{ M}^{-1}\text{cm}^{-1}$. The correction factor 0.16 was included to compensate for absorption of the coumarin label at 280 nm (personal communication, Molecular Probes, Inc.).

Calculation of Peptide Diameter. The effective spherical diameter of the peptide was calculated using the relation [Creighton, p.189 (1993)].

$$\sigma = 2 \left(\frac{3M\bar{v}}{4\pi N_A} \right)^{1/3} \quad (\text{A.4})$$

where the peptide molecular weight, M , is 2969 g/mol, and the partial specific volume of the peptide, \bar{v} , is 0.7 ml/g. N_A is Avogadro's number.

Accuracy of Fit. Figures 2 through 4 show the best-fit curves to the data using equation (13). When there is no sodium present, as in Figure 2, the fit is excellent. R^2 values are greater than 95%. At increasing salt concentration, the fit becomes less accurate. The R^2 values for curves at pH 4 fall from 83% to 76% as the sodium-chloride concentration increases from 0.1 to 1.0 M (see Figure 3). At pH 5, R^2 ranges from 75% to 92%. Lowered accuracy of fit may be due to formation of higher aggregates than dimers at higher ionic strengths. Our model does not account for the existence of such oligomers.

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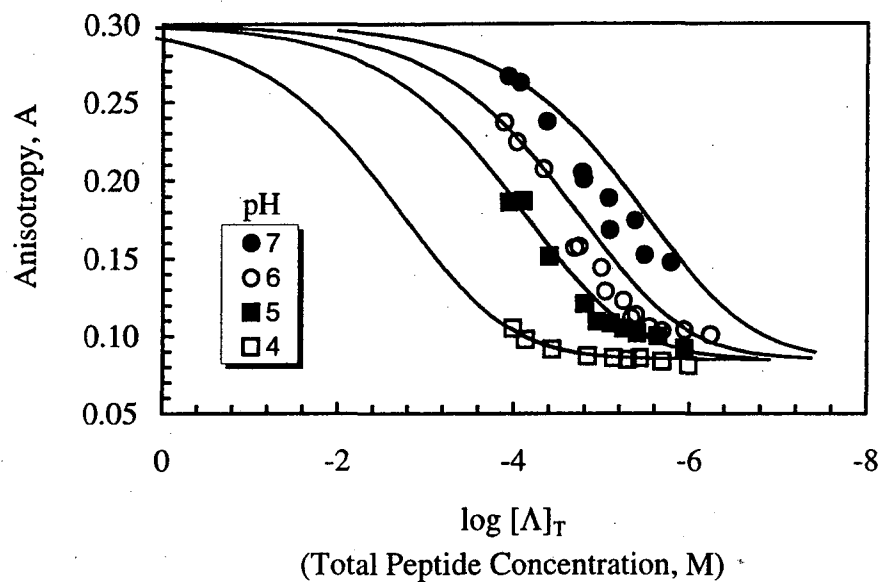


Figure 1: Anisotropy measurements for T4 LYS(11-36) in 25-mM sodium phosphate buffer as a function of pH. NaCl concentration is zero. Symbols represent experimental data at 18°C. Solid lines represent extrapolation to infinite dilution (A_f) and to saturation (A_b).

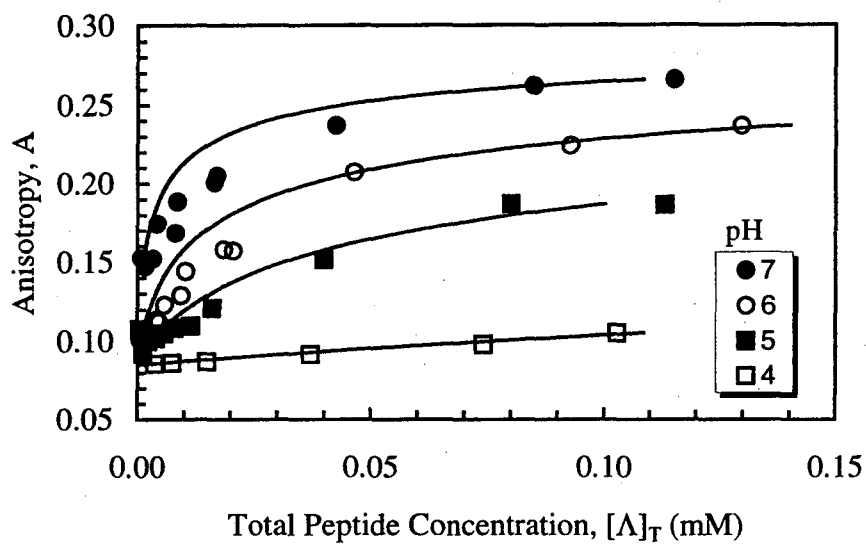


Figure 2: Anisotropy measurements for T4 LYS(11-36) in 25-mM sodium phosphate buffer as a function of pH. NaCl concentration is zero. Symbols represent experimental data at 18°C. Solid lines represent the best-fit curves using equation (13).

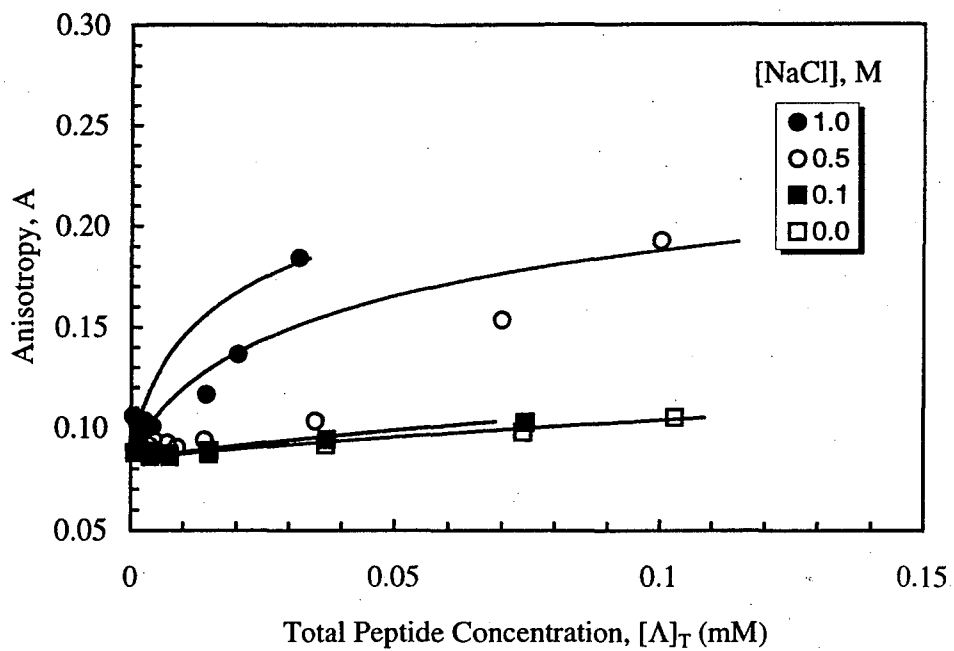


Figure 3: Anisotropy measurements for T4 LYS(11-36) in 25-mM sodium phosphate buffer at pH 4 as a function of NaCl concentration. Symbols represent experimental data at 18°C. Solid lines represent the best-fit curves using equation (13).

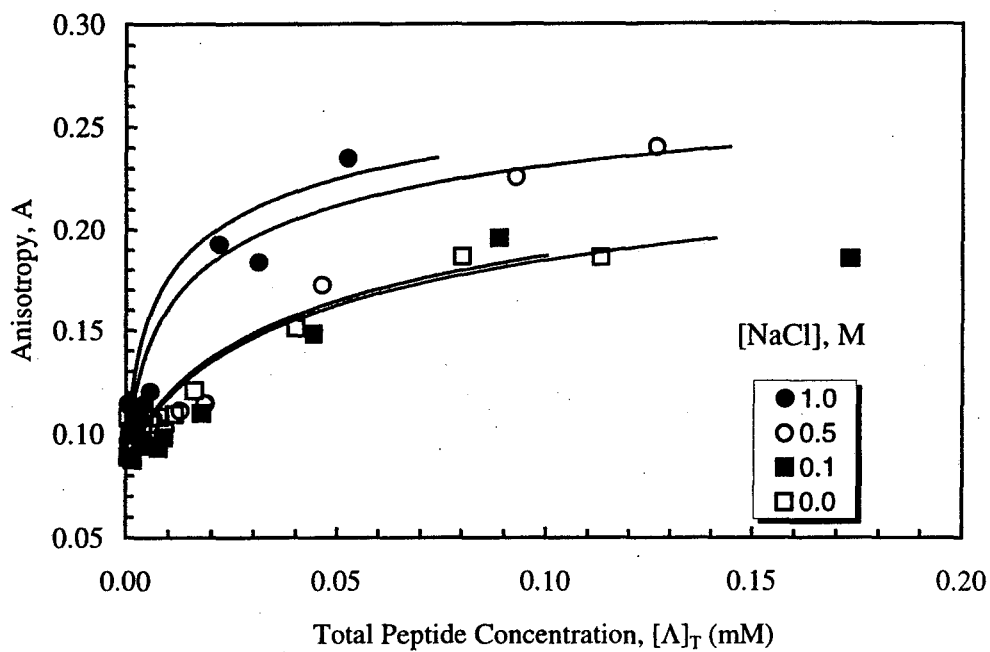


Figure 4: Anisotropy measurements for T4 LYS(11-36) in 25-mM sodium phosphate buffer at pH 5 as a function of NaCl concentration. Symbols represent experimental data at 18°C. Solid lines represent the best-fit curves using equation (13).

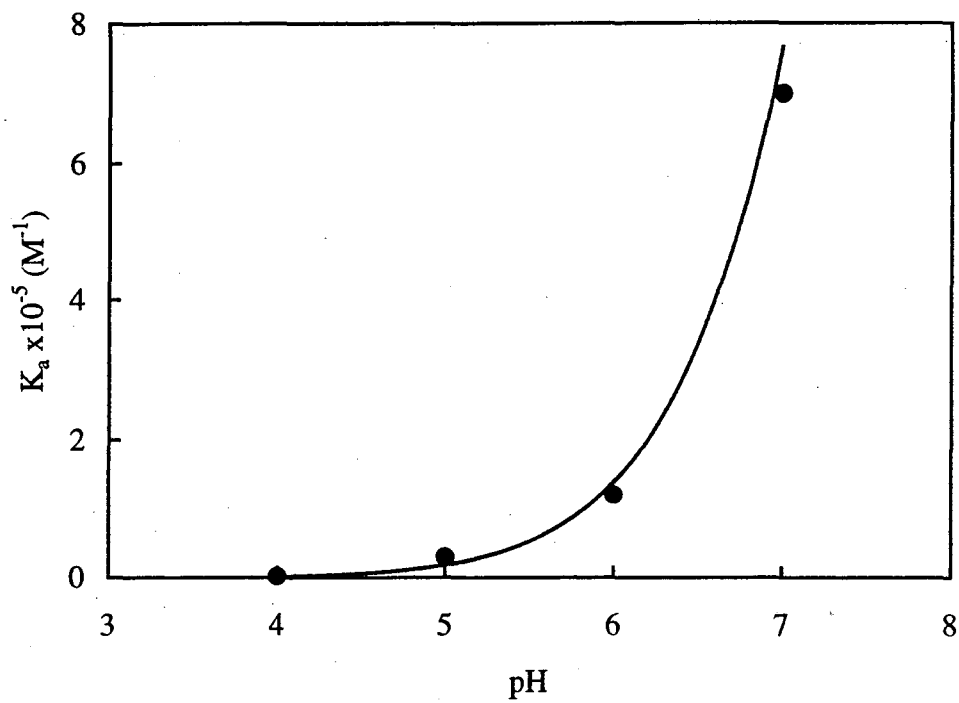


Figure 5: Association constant, K_a , for dimerization of T4 LYS(11-36) at 18°C as a function of pH. NaCl concentration is zero.

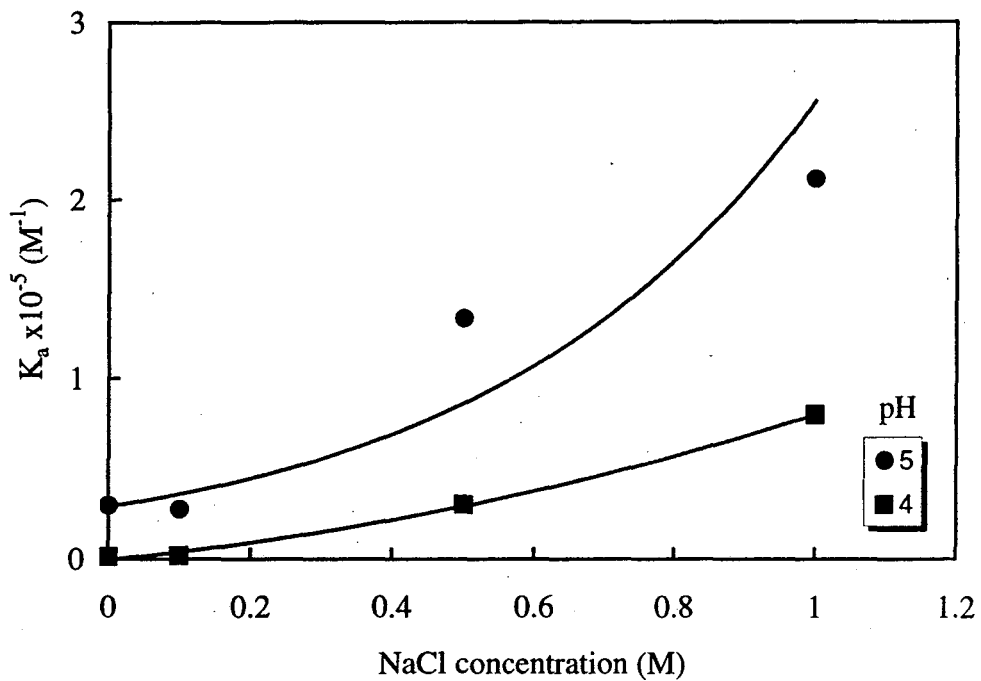


Figure 6: Association constant, K_a , for dimerization of T4 LYS(11-36) at 18°C as a function of NaCl concentration.

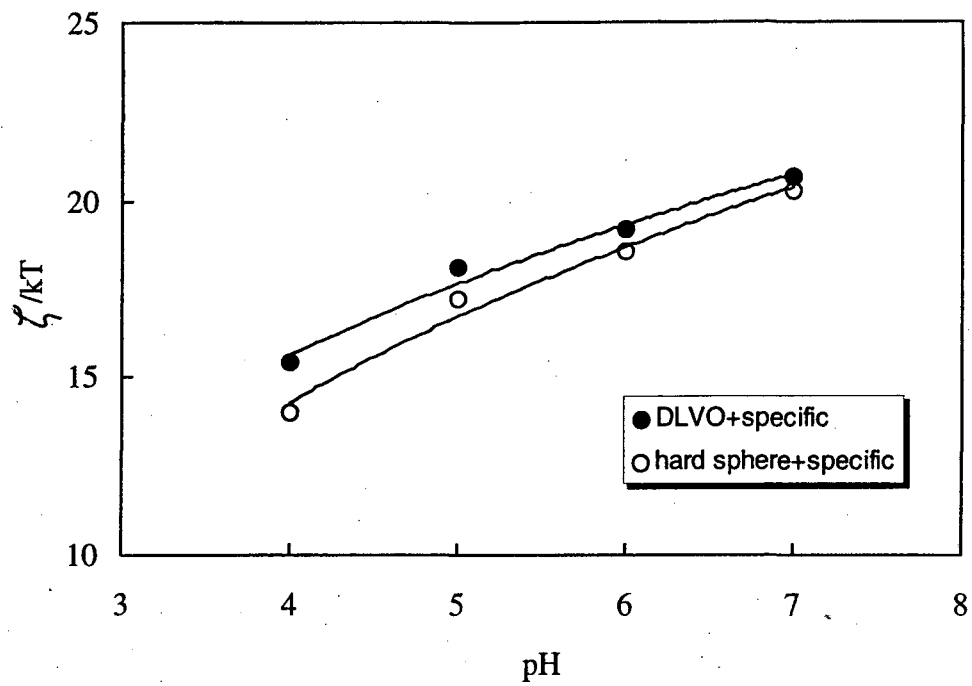


Figure 7: Square-well depth, ζ , as a function of pH. NaCl concentration is zero.

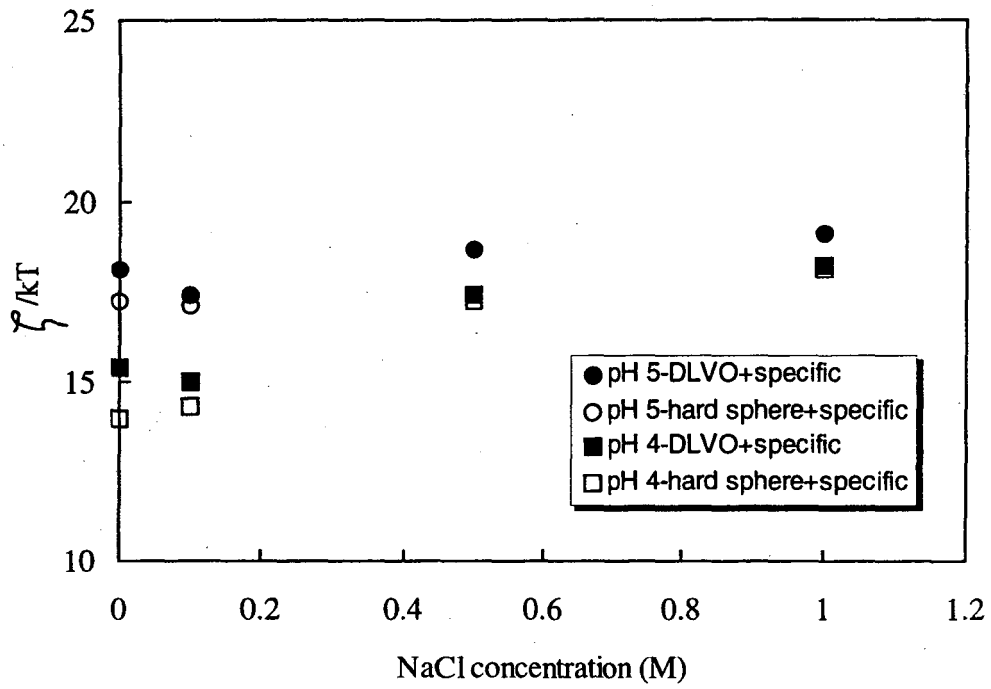


Figure 8: Square-well depth, ζ , as a function of NaCl concentration.

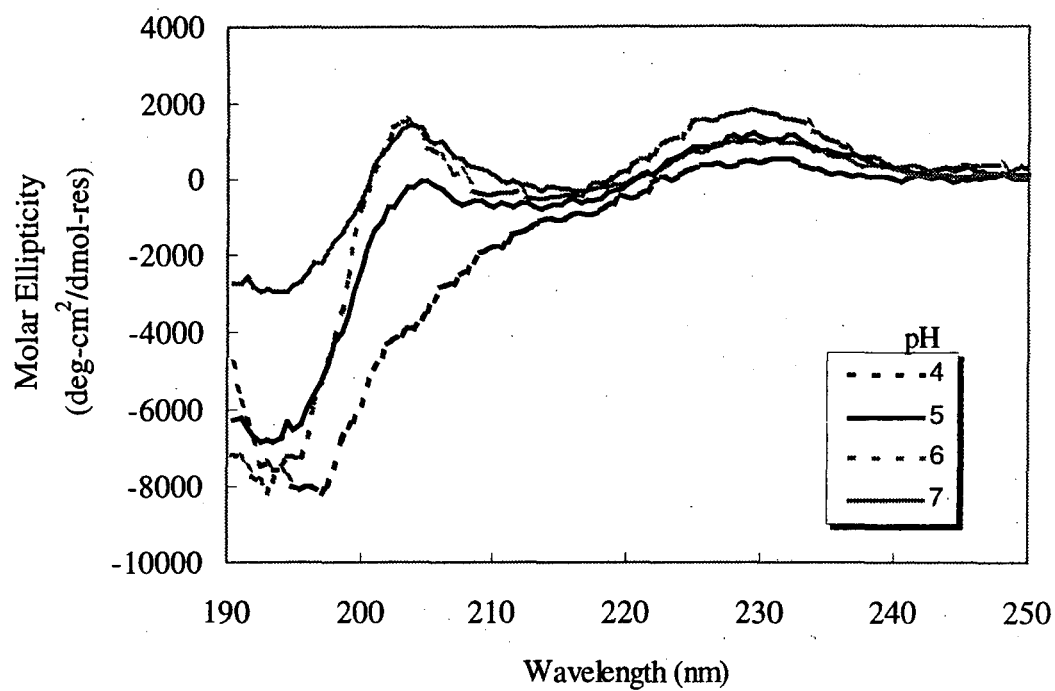


Figure 9: Far-UV CD spectra of the T4 LYS(11-36) peptide in 5-mM sodium phosphate buffer at 25°C.

Table 1: Net electric charge of T4 LYS(11-36) as a function of pH (calculated from free-amino-acid titration in 0.1 M KCl).

<i>pH</i>	4	5	6	7
<i>Net charge</i>	+3.1	+2.5	+2.1	+1.7

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