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Contribution of a Pyrene Fluorescence Probe to the Aggregation Propensity of Polypeptides

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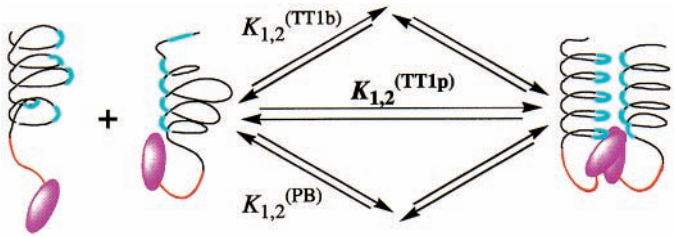
ABSTRACT



Two synthetic polypeptides, TT1p and TT1b, have been used in comparative aggregation equilibrium studies. The findings reveal that a single alkylpyrene moiety in TT1p contributes about 30% of the polypeptide dimerization energy in aqueous media. This result not only is informative with regard to the aggregation properties of these particular photoactive polypeptides but also provides a quantitative understanding of the limitations on the use of pyrene chromophores as emission probes.

Pyrene derivatives are ubiquitous photophysical probes of supramolecular structural properties[1] and protein conformational changes and aggregation phenomena.[2] Alkylpyrene moieties have also been incorporated in synthetic peptides, and their fluorescence properties have been applied to the determination of the conformation of peptide strands,[3] as well as the assembly of tertiary and quaternary structures such as helix bundles.[4] The convenience of using this type of photophysical probe in structural studies rests with the reliability of determining the proximity of alkylpyrene chromophores in a macromolecular assembly by monitoring the alterations in emission spectra. When pyrenes are separated as monomers, chromophores display an emission band with distinct vibrational structure between 370 and 430 nm; a broad vibrationless (excimer) band centered between 450 and 500 nm is observed when pyrenes are in close proximity.[5] In addition, the ground-state aggregation of the chromophores leads to perturbation in the UV/vis absorption spectrum, i.e., a red shift of the absorption maximum and a decrease in molecular extinction coefficient are observed.[6,7]

The application of pyrene chromophores for analysis of aggregation phenomena, however, ought to be approached

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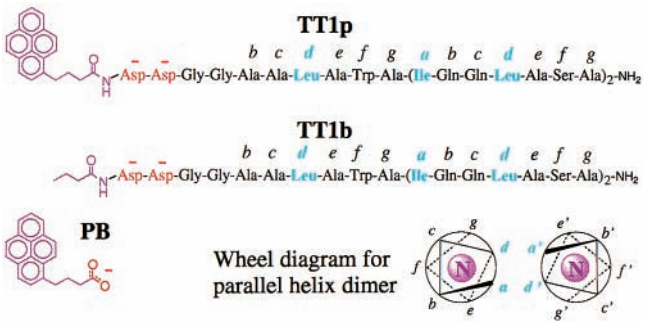
with caution, because they are not passive fluorescence probes. For example, it has been qualitatively demonstrated that alkylpyrenes tend to alter the aggregation properties of polypeptides that carry the pyrene fluorescent label.[7,8] The dimerization energy of an alkylpyrene derivative, 4-(1-pyrene)butanoate (PB), in aqueous media has been measured to be −2.9 kcal/mol.[9] The current article presents a quantitative analysis of the dimerization properties of a pyrene-containing polypeptide, TT1p,[7] yielding results that allow the partitioning of aggregation energy in terms of contributions involving the interaction of pyrene moieties and those related to the polypeptide chains. For the purpose of the investigation, mathematical treatments for concentration-dependent emission and circular dichroism (CD) data have been developed.

TT1p is a 24-residue amphipathic helical polypeptide carrying an alkylpyrene chromophore at the N-terminus, introduced as a pyrenebutyramide. The design features and the preparation of TT1p have been previously described.[7] To investigate the role of the pyrene moiety in the aggregation properties of TT1p, an analogous polypeptide lacking the pyrene chromophore, TT1b, has been prepared for comparison. For this model, butanoic acid was used for capping the N-terminus (Scheme 1). Both polypeptides carry

Scheme 1



negative charges at their N-termini via aspartate residues. The results obtained for TT1p and TT1b are, therefore, plausibly compared with the aggregation data obtained for PB, a negatively charged pyrene derivative that lacks a peptide chain (Scheme 1).[9] At high concentrations, TT1p has been shown to exhibit complex aggregation behavior, i.e., a dimer−octamer equilibrium is observed in the lower micromolar concentration range.[7] The present study was conducted at submicromolar concentrations where the polypeptide aggregation equilibrium is predominantly monomer to dimer.[10]

The emission spectra recorded at room temperature for TT1p in aqueous media, for a 3 orders of magnitude polypeptide concentration range (8 nM to 8 μM), are shown in Figure 1. As expected, at higher concentrations the

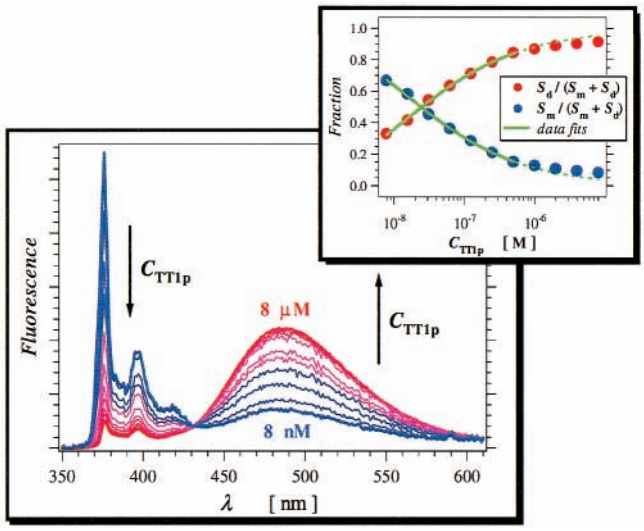


Figure 1. Concentration dependence of alkylpyrene emission for TT1p in aqueous media (1 mM phosphate buffer, pH 8) (λ_{ex} = 333 nm). Inset: alterations in the normalized integrated monomer, S_m, and dimer, S_d, emissions as functions of total polypeptide concentration, C_{TT1p}.

polypeptide exhibits green fluorescence due to the dominance of the broad emission band centered at 485 nm, which is traditionally ascribed to pyrene chromophore aggregates.[1−5] With dilution to less than 0.5 μM, the relative intensity of the pyrene aggregate emission band decreases while a concurrent growth of pyrene monomer fluorescence with maxima at 376 and 397 nm is observed. Polypeptide samples are blue-fluorescent in the low-nanomolar concentration range. These changes in the emission properties are presented in the inset of Figure 1 as ratios of the areas under the monomer, S_m, and the aggregate, S_d, spectra, respectively. (The values for S_m and S_d were obtained by numerical integration of the monomer and aggregate emission bands, respectively, $S = \int \mathbf{F}(\bar{\nu})\mathrm{d}\bar{\nu}$; see Supporting Information for details.)

For the low concentration range, the observed alterations in emission can be ascribed to the polypeptide monomer−dimer equilibrium, 2 (pyrene) $\rightleftarrows$ (pyrene)$_2$, having a dimerization constant

$$K_{1,2}^{(\mathrm{TT1p})} = \frac{[(\mathrm{pyrene})_2]}{[(\mathrm{pyrene})]^2} \qquad (1)$$

where [(pyrene)$_2$] and [(pyrene)] are the equilibrium concentrations of pyrene chromophore dimer and monomer, respectively, representing the corresponding TT1p dimer and monomer concentrations. The total polypeptide concentra-

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(10) The tetramerization constant for the dimer−octamer equilibrium of TT1p in aqueous media has been measured by equilibrium sedimentation analysis:[7] $K_{2,8} = 3.3 \times 10^{17}$ M^{-3}. This value indicates that at 40 μM concentration, about 90% of the polypeptide will be aggregated as an octamer and 10% as a dimer, while at 1 μM, 90% will be in the form of a dimer and only 10% as an octamer.

tion, C_{TT1p}, can be readily represented in terms of total chromophore concentration, C_{pyrene}:

$$C_{TT1p} = C_{pyrene} = [(pyrene)] + 2[(pyrene)_2] \qquad (2)$$

Therefore, the ratio between the dimer and monomer emissions, $R_S = S_d/S_m$, can be expressed as a function of the total polypeptide concentration:

$$R_S = \frac{-b + \sqrt{b^2 + 4aC_{TT1p}}}{2a} \qquad (3)$$

where

$$a = \frac{2}{K_{1,2}^{(TT1p)}} f^2(\lambda_{ex}), \quad b = \frac{1}{K_{1,2}^{(TT1p)}} f(\lambda_{ex})$$

and $f(\lambda_{ex})$ is a function that is related to the photophysical properties of the alkylpyrene monomer and dimer (see Supporting Information for details) and is independent of the concentration of the species. Therefore, $K_{1,2}^{(TT1p)}$ and $f(\lambda_{ex})$ were the two fitting parameters used in the subsequent data analysis. Fitting the TT1p emission data obtained for the 8 nM to 0.5 μM concentration range using eq 3 (Figure 1, inset)[11] yielded $\ln(K_{1,2}^{(TT1p)}) = 18 \pm 0.3$ ($K_{1,2}^{(TT1p)} = 6.6 \times 10^7$ M^{-1}), values that correspond to an aggregation free energy of -10 ± 0.2 kcal/mol (calculated for 20 °C).

The aggregation properties of the polypeptide without the alkylpyrene chromophore, TT1b, were examined using a concentration-dependent CD spectroscopy method.[12,13] The strong concentration dependence of the CD properties of TT1b in the range between 400 nM and 80 μM is shown in Figure 2a. The data are consistent with a significant decrease in the polypeptide helicity on lowering total peptide concentration, C_{TT1b}. In Figure 2b the same concentration dependence is presented in terms of the ratio between the measured ellipticities, $R_\theta = \theta_{222}/\theta_{208}$, at the two minima at 222 and 208 nm, CD spectral features that are characteristic of a right-handed α-helix.[14] It is reasonable to attribute this change in the secondary stricture to self-aggregation of the polypeptide, where for a one-step process, $nP \rightleftarrows P_n$,[15,16] the equilibrium constant is given by the relation

$$K_{1,n}^{(TT1b)} = \frac{[P_n]}{[P]^n} \qquad (4)$$

where [P] and [P_n] are the equilibrium concentrations of the TT1b monomer and aggregate, respectively.

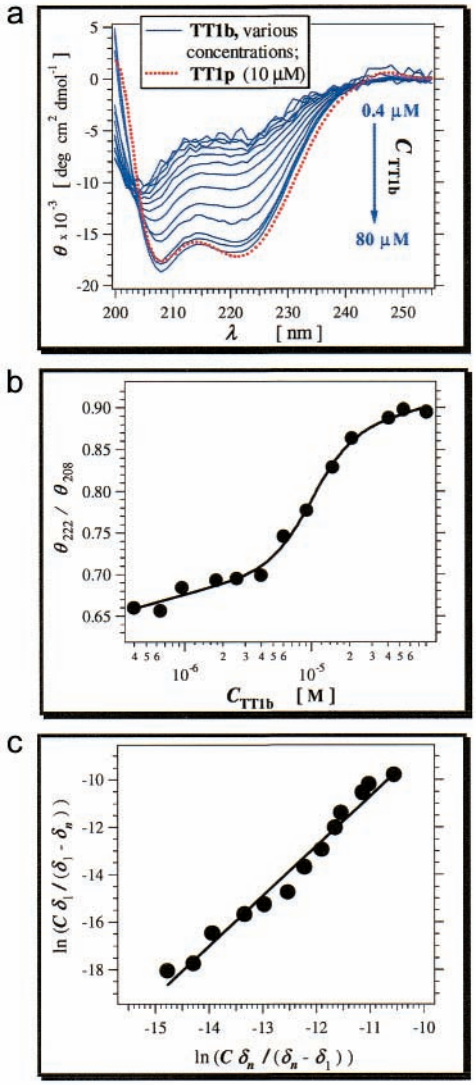

Figure 2. Concentration dependence of the circular dichroism properties of TT1b in aqueous media (1 mM phosphate buffer, pH 8): (a) CD spectra of TT1b at various concentrations, in comparison with the CD spectrum of TT1p; (b) alteration in the ratio between the molar ellipticities at 222 and 208 nm for TT1b, as a function of total polypeptide concentration; (c) linear analysis of the concentration-dependent CD for TT1b.

For further data analysis, it is necessary to introduce the parameters $\delta_1 = \theta_{222}^{(1)} - R_\theta\theta_{208}^{(1)}$ and $\delta_n = \theta_{222}^{(n)} - R_\theta\theta_{208}^{(n)}$ where the superscripts (1) and (n) indicate the ellipticities of the monomer and the aggregate, respectively, obtained by data extrapolation to zero and infinite polypeptide concentrations. As a result, the relationship between the total polypeptide concentration and its CD properties can be expressed in the following form (for derivation, see Supporting Information):

(11) The relations between R in eq 3 and the relative emissions on the inset of Figure 1 are $S_m/(S_m + S_d) = 1/(1 + R_S)$ and $S_d/(S_m + S_d) = R_S/(1 + R_S)$.

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(16) More complex aggregation schemes have been used as a model; however, the data analysis did not produce any statistical significance over the one-step process (see Supporting Information for details).[15]

$$\ln\left(\frac{C_{\mathrm{TT1b}}\delta_1}{\delta_1-\delta_n}\right)=n\ln\left(\frac{C_{\mathrm{TT1b}}\delta_n}{\delta_n-\delta_1}\right)+\ln(K_{1,n}^{(\mathrm{TT1b})})+\ln(n) \quad (5)$$

The linear data fit using eq 5 is shown in Figure 2c. From the slope, the parameter reflecting the state of aggregation, $n = 2.1 \pm 0.1$, could be obtained. The results support the monomer−dimer equilibrium model. In addition, the intercept of the fit yielded the aggregation constant, $\ln(K_{1,n}^{(\mathrm{TT1b})}) = \ln(K_{1,2}^{(\mathrm{TT1b})}) = 12 \pm 1.2$ ($K_{1,2}^{(\mathrm{TT1b})} = 1.6 \times 10^5\ \mathrm{M}^{-1}$),[17] values that reveal a free energy of dimerization of -7.0 ± 0.7 kcal/mol.

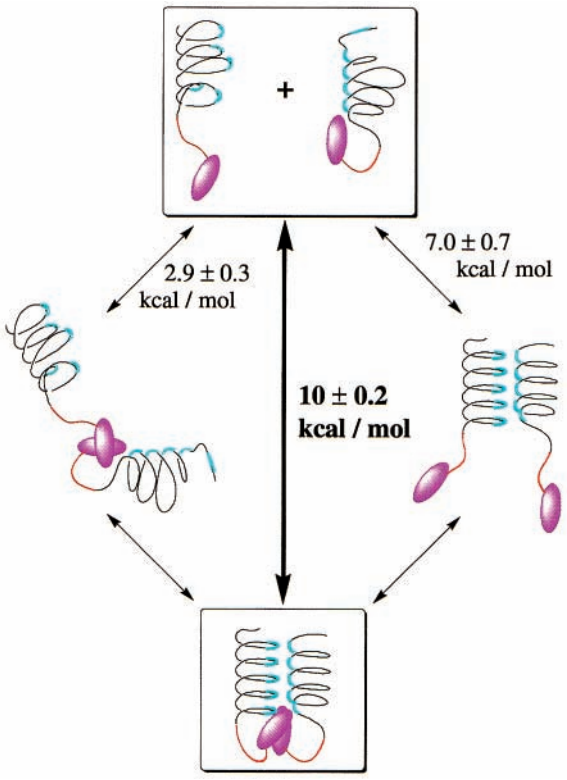
The results obtained suggest that there is no significant cooperativity involving peptide−peptide and chromophore−chromophore interactions. This feature of the TT1p system can be treated according to the method of Jencks,[18] who developed an expression for "connection" free energy as follows:

$$\Delta G^{(\mathrm{S})} = \Delta G_{\mathrm{Pep}} + \Delta G_{\mathrm{Chrm}} - \Delta G_{\mathrm{P-C}} \quad (6) \qquad (6)$$

where $\Delta G_{\mathrm{Pep}} = -RT\ln(K_{1,2}^{(\mathrm{TT1b})})$, $\Delta G_{\mathrm{Chrm}} = -RT\ln(K_{1,2}^{(\mathrm{PB})})$, and $\Delta G_{\mathrm{P-C}} = -RT\ln(K_{1,2}^{(\mathrm{TT1p})})$. The calculated values for $\Delta G^{(\mathrm{S})}$, related to TT1p dimerization, fall in the range between 0.1 and 1 ($\pm$ 0.8) kcal/mol, small numbers that do not exceed 10% of the total binding energy, $\Delta G_{\mathrm{P-C}}$ (see Supporting Information for details). Thus, within the error limits, the dimerization energies for the chromophore, ΔG_{Chrm}, and the polypeptide, ΔG_{Pep}, are additive to yield the total binding energy, $\Delta G_{\mathrm{P-C}}$. This result indicates that the chromophores interact relatively independently from the peptide chains, a phenomenon that can be ascribed to the flexibility of chains that link the pyrene moieties and the polypeptides (Scheme 1).

In summary, the energy of dimerization for TT1p in aqueous media is about 3 kcal/mol more negative than the dimerization energy for TT1b, an indication that the alkylpyrene chromophore attached to the N-terminus of the polypeptide (Scheme 1) contributes about 30% of the TT1p aggregation energy. This result is in excellent agreement with studies conducted on PA that yielded for the chromophore itself a dimerization free energy of -2.9 ± 0.3 kcal/mol.[9] A graphic representation of this aggregation energy partition is shown in Scheme 2. Assuming that the leucine zipper interaction is the major contributing force for aggregation

Scheme 2



of the polypeptide chains,[19] the average contribution of each of the five Leu(Ile) pairs toward the dimerization energy will be about −1.4 kcal/mol. That is, the interaction energy between pairs of amino acid chains in the leucine zipper is about half that of the interaction of alkylpyrene chromophores. Another impact of this result is the recognition that alkylpyrene chromophores are not an ideal choice as passive fluorescence probes, at least in terms of monitoring binding equilibria, because they provide an energetic bias favoring the interaction of proximate pyrenes.

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Supporting Information Available: Experimental details and derivations of eqs 3 and 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The results from the analysis of the CD data are in excellent agreement with equilibrium ultracentrifugation measurements; e.g., sedimentation analysis yielded an aggregation constant of $1.8 \times 10^5\ \mathrm{M}^{-1}$ for TT1b monomer−dimer equilibrium under identical conditions.[7]

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