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Evaluation of the Physical Stability of the EC5 Domain of E-Cadherin: Effects of pH, Temperature, Ionic strength, and Disulfide Bonds

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Abstract

The development of protein drugs has been hampered by difficulties in formulating them due to their inherent chemical and physical stability, which could generate problems during the late stages of development. Thus, a basic understanding of the effect of structural features on the physicochemical stability of proteins can provide fundamental solutions to the formation of proteins. In this work, the physical stability of the EC5 protein under variable pH, temperature, and ionic strength and the role of the disulfide bond on the physical stability of EC5 were evaluated. All spectroscopic measurements were integrated in empirical phase diagrams, and these diagrams showed the stable and unstable regions of EC5. The native EC5 is more stable at high than at low ionic strength in a wide pH range during temperature elevation to 70°C. The empirical phase diagrams also show that the reduced EC5 has lower stability than the parent EC5. The reduced EC5 has secondary structure only at pH 3 and 4 and is unfolded at other pH values. Finally, the reduced EC5 rapidly forms a precipitate at pH 4 and 5 upon heating. In conclusion, this study shows that ionic strength and the presence of the disulfide bonds are critical for the stability of EC5.

Keywords

EC5; protein stability; CD; intrinsic fluorescence; UV absorbance; optical density; phase diagram

INTRODUCTION

Recent success in utilizing proteins (*i.e.*, monoclonal antibodies, hormones, and cytokines) as therapeutic agents has stimulated the development of new and rapid methods to formulate these types of agents.¹ Although proteins have high binding affinity and selectivity for their target receptors *in vivo*, the development of these molecules has been hampered by the difficulty in finding a formulation that has a long shelf-life. This difficulty is partly due to the potential physical and chemical degradation events displayed by proteins in solution as well as in solid formulations.^{2–4}

Physical degradation such as unfolding, aggregation, and precipitation in solution occurs in part due to the presence of secondary and tertiary structures in proteins.^{4,5} Solution conditions (pH, buffer), physical agitation, and protein interactions with hydrophobic surfaces (*i.e.*, protein–air and protein–container interactions) can all accelerate the physical

degradation of proteins. Due to the nature of the functional groups in amino acid side chains, proteins can also chemically degrade via several different reactions, including deamidation, β -elimination, oxidation, and disulfide exchange.^{4,6,7} It has been suggested that there can be a cause and effect relationship between chemical and physical degradation; for example, chemical degradation may induce structural changes in proteins that lead to physical degradation and *visa versa*. Physical and chemical degradation can both produce a loss of biological activity or changes in antigenicity that result in pathological immune responses. Thus, a better understanding of the physicochemical properties of proteins can be used to devise methods to improve protein stability in pharmaceutical formulations.

EC5 is the fifth extracellular domain of a calcium-dependent protein called E-cadherin; it has an immunoglobulin structure with seven β -sheet strands and two disulfide bonds (Fig. 1).⁸ The EC5 domain of E-cadherin has an important role during T-cell adhesion to epithelial cells via $\alpha_E\beta_7$ integrins on the surface of T cells.⁹ Our previous studies suggest that the EC5 protein can modulate the calcium-dependent E-cadherin-mediated single cell adhesion to cell monolayers of epithelial cells. In addition, E-cadherin-derived peptides have been shown to enhance the permeation of marker molecules via the paracellular pathways of Madin-Darby Canine Kidney (MDCK) cell monolayers.^{10,11} These peptides have also been shown by spectroscopic methods (*i.e.*, intrinsic fluorescence, far-UV circular dichroism (CD), FTIR, and NMR) to bind to the EC5 domain.¹² Thus, the EC5 protein may be a good candidate for blocking T-cell adhesion to epithelial cells during inflammation. In addition, the presence of two disulfide bonds in EC5 makes it a good model for studying the role of these disulfide bonds in its physical stability.

In this work, the effect of disulfide bonds on the physical stability of EC5 was evaluated under different conditions (*i.e.*, pH, temperature, and ionic strength (*I*)). Changes in the secondary structure of both native and reduced EC5 during temperature elevation were monitored by CD spectrometry. Tertiary structural changes as a function of temperature were detected by intrinsic fluorescence and UV absorbance spectroscopies. The aggregation and precipitation properties of this protein were observed by monitoring changes in optical density. The data from all these measurements were integrated into empirical phase diagrams to describe the global changes in protein structure over a wide range of different conditions. The results show that disulfide bonds play an important role in the physical stability of EC5 and provide a comprehensive physical picture of the stability of this important cadherin domain.

EXPERIMENTAL METHODS

EC5 Preparation

Recombinant EC5 cloned in an pET24d vector was over-expressed and purified using the method previously described.¹² The final, pure EC5 domain was dialyzed using Biomax membranes (Millipore, Billerica, MA) with a 10 kDa cutoff against a 20 mM phosphate, 20 mM citrate buffer at pH 3–8 with an ionic strength (*I*) equal to 1.0 or against the same buffer with an ionic strength (*I*) of 0.1. The ionic strength was controlled by the addition of an appropriate amount of sodium chloride. Reduced EC5 was dialyzed using the same membrane against a 20 mM phosphate, 20 mM citrate buffer containing 2.5 mM DTT at pH 3–8 at an ionic strength (*I*) of 0.1.

Stability of EC5 Secondary Structure

The secondary structural stability of EC5 under different conditions was monitored by CD employing a J-810 CD spectrometer (Jasco, Easton, MD) with spectra obtained from 250 to 200 nm. The solution of EC5 (400 μ L, 20 μ M EC5) was placed in a 0.1-cm pathlength

quartz cuvette for data collection, and the contribution of the buffer was subtracted from the spectrum. The thermal unfolding of EC5 was monitored at 230 nm by varying the temperature from 10 to 85°C with data collected in 2.5°C increments. A 5-min equilibration was performed prior to each data collection. The melting temperature of EC5 under each condition was estimated by sigmoidal fit in Origin 7.0 (OriginLab Corporation, Northampton, MA).

Stability of EC5 Tertiary Structure

Intrinsic Fluorescence Spectroscopy—Intrinsic fluorescence emission scans were collected with a QuantaMaster spectrofluorometer (PTI, Monmouth Junction, NJ). A solution of 20 μ M EC5 (1.5 mL) was placed in a 1-cm pathlength quartz cuvette, and the spectra were collected at 10–85°C in 2.5°C increments. A 5-min equilibration was performed before collection of each spectrum. The excitation wavelength was set at 295 nm and emission was monitored from 305 to 455 nm. The excitation and emission slits were set at 4 nm. All spectra were analyzed using Origin 7.0.

UV Absorbance Spectroscopy—UV absorbance spectra were recorded using a Hewlett-Packard 8453 UV–vis diode array spectrophotometer (Agilent, Palo Alto, CA). A 1-cm pathlength quartz cuvette containing 0.5 mL of 20 μ M EC5 was used during all experiments. Spectra were collected over the temperature range of 10–85°C at 2.5°C intervals using a 5-min equilibration period before data acquisition. Agilent UV–Visible Chemstation software was used to calculate the second derivative spectra. A 9-point data filter and 5th degree Savitzky-Golay polynomial was used during the calculation. The spectra were fit to a cubic function, in which each raw data point contains 99 interpolated points. The resulting spectra have an effective resolution of 0.01 nm and are plotted in Origin 7.0.^{13,14} The optical density of the solution was also monitored simultaneously during the UV melting experiments by recording the value at 350 nm.

Empirical Phase Diagram

To better interpret the complex data sets from the multiple analytical techniques (far-UV CD, intrinsic fluorescence, and second-derivative UV absorbance) that were employed to characterize the physical properties of EC5 under various solution conditions, empirical phase diagrams were constructed employing an RGB color-coding-based system using Matlab (The Mathworks, Natick, MA), in which different physical states of EC5 could be assigned to substantially uniform colored regions and correlated with related structural data. The theory of the generation of empirical phase diagrams has been described in detail previously.¹³

RESULTS

Stability of EC5 Secondary Structure

The molecular model of EC5 (Fig. 1) shows that this protein has seven β -sheet strands, and the CD spectra indicate that EC5 has a high content of the β -sheet structure. The CD spectra of EC5 are dominated by a positive peak around 230 nm, which is presumably due to a combination of the absorptions of the aromatic residues and disulfide bonds. The negative ellipticity below 220 nm and the shoulder at 210 nm presumably reflect the presence of β -trefoil structure.¹⁵ At $I = 1.0$, the secondary structure of EC5 appears very similar over the entire pH range examined (pH 3–8), suggesting that pH does not have a significant effect on the stability of the secondary structure of EC5 at this ionic strength (data not shown). In contrast, the secondary structure of EC5 is more sensitive to pH changes at a lower ionic strength of 0.1 (Fig. 2A). The pH-dependent CD spectra of EC5 at $I = 0.1$ can be separated into two classes. One is seen at pH 3–5 and the other at higher pH (6–8) with the spectra at

lower pH exhibiting increased negative ellipticity below 220 nm. The effect of ionic strength on the secondary structure stability of EC5 is due to the effect of salt on charge–charge and charge–dipole interactions on the protein surface (see Discussion Section).

A more dramatic structural change is observed upon reduction of the disulfide bonds. The positive peak at 230 nm disappears, indicating the loss of a well-folded structure (Fig. 2B). At pH 3, EC5 may still maintain some secondary structure although the spectrum appears very different than that of the non-reduced state. At pH 4, most of the protein ellipticity is lost due to its precipitation. At pH 5–8, the circular dichroism of reduced EC5 is dominated by disordered structure as shown by the minima below 200 nm (Fig. 2B).

Because the 230 nm maximum is the dominant signal in the EC5 spectrum, the molar ellipticity at 230 nm was monitored as a function of temperature to probe the thermal stability of the domain. To perform a more accurate comparison of the data under each condition, the relative molar ellipticity of each peak was normalized to the initial value at 10°C (Fig. 2C and D). At $I = 1.0$, there was no significant difference in the melting temperature (T_m) of native EC5 from pH 4 to 8; however, the T_m of EC5 is about 2°C lower at pH 3 than at pH 4 (data not shown). At $I = 0.1$, the overall T_m is approximately 10°C lower (Fig. 2C) than at the ionic strength of 1.0. In the case of the reduced EC5 domain (Fig. 2D), the positive peak at 230 nm disappears and the well-defined thermal transition becomes undetectable from pH 5 to 8. This is no doubt due to the absence of folded structure in reduced EC5 at pH 5–8, as shown in Figure 2D. Although the CD spectrum at pH 3 indicates the presence of some secondary structure, no clear transition is observed at this pH value. In addition, gross precipitation of reduced EC5 causes the CD spectra to be very noisy at elevated temperature. In summary, the secondary structure of EC5 is more stable at higher ionic strength (1.0) than at lower ionic strength (0.1). Reducing the protein's disulfide bonds dramatically reduces its thermal stability.

Stability of EC5 Tertiary Structure

Intrinsic Fluorescence Spectroscopy—Intrinsic fluorescence spectroscopy was used to monitor the tertiary structural changes in EC5 by observing microenvironmental changes of the single EC5 Trp residue. The effect of pH on the thermal unfolding of EC5 was examined by plotting relative emission peak intensities *versus* temperature. There is no major difference in the relative peak intensities over the entire pH range examined when $I = 1.0$ (data not shown); this is consistent with the secondary structure-sensitive data from CD. At $I = 0.1$ and pH 3 and 4, the temperature-dependent relative fluorescence intensities manifest small inflections around 60–70°C compared to data at other pH values (Fig. 3A), suggesting a minor change in tertiary structure. Reduced EC5 displayed clear transitions around 30°C at pH 4 and 35°C at pH 5 (Fig. 3B). The dramatic slope changes suggest significant structural changes in EC5 due to the formation of aggregates and the observed white precipitates.

Emission peak position shifts were also used to monitor the temperature-induced unfolding of the native and reduced EC5 domains. The emission peak position changes of the single Trp residue in native EC5 show a similar trend with red shifts upon temperature elevation (Fig. 3C), indicating that the indole side-chain is exposed to a more polar environment as the temperature increases. In other words, as the temperature is elevated, the structure of EC5 is gradually unfolded, making the hydrophobic core more accessible to the aqueous environment. In general, the thermal transitions over the entire pH range examined are approximately 10°C higher at $I = 1.0$ than at $I = 0.1$. Reduced EC5 shows a red-shifted thermal transition only at pH 3 (Fig. 3D). However, this transition occurs about 15°C lower than that of native EC5. This is consistent with the secondary structural studies from CD spectroscopy. At pH 4 and 5 (Fig. 3D), the emission peaks are detectable only until 30 and

35°C, respectively. Beyond those points, the emission peaks are completely obscured by the dominant light scattering peaks. The relatively low solubility seen at pH 4 and 5 occurs because these pH values are close to the *pI* (4.73) of EC5. No thermal transitions are observed at pH 6–8 (Fig. 3D), suggesting a lack of well-defined tertiary structure at these pH values.

UV Absorbance Spectroscopy—EC5 possesses six aromatic residues: three Phe, two Tyr, and one Trp. Monitoring changes in the microenvironments around these residues upon temperature elevation using UV absorption spectroscopy has the potential to provide a more global picture of the thermal behavior on tertiary structure than the analysis of Trp spectra alone. After collecting high resolution UV absorbance spectra, the second derivative of each spectrum was calculated for resolution enhancement. EC5 shows six negative peaks in the second derivative UV spectrum from Phe (253, 259, and 266 nm), Tyr (275 nm), Tyr/Trp (284 nm), and Trp (291.5 nm).¹² Here, the behavior of three of the six peaks is presented because the rest of the data is redundant. These three second derivative UV absorbance peak positions as a function of temperature under various pH conditions are plotted to detect potential tertiary structure changes in EC5.

At an ionic strength of 1.0, the first UV peak (253 nm) from the Phe residues shows a constant red shift at pH 3–6 with no evidence of any inflection points (data not shown). At pH 7 and 8, although not very dramatic, evidence for weak transitions can be observed between 65 and 85°C. When the ionic strength is reduced to 0.1, more visible transitions can be seen in EC5 between 55 and 75°C at pH 4–8, although no obvious transition is found at pH 3 (Fig. 4A). This suggests that the tertiary structure around the Phe residues is more stable at high than at low ionic strength. During the observed transitions, the blue shift seen between 55 and 75°C indicates that the Phe residues are becoming more exposed to the polar solvent upon conformational alteration. The Phe residues in the reduced form of EC5 are not sensitive to temperature changes at pH 3–7 (Fig. 4B). At pH 8, however, there is an observable transition around 42°C. After this transition, the spectrum appears to be very noisy, due to gross precipitation.

The next UV peak (259 nm) from the Phe residues does not shift upon temperature elevation over the entire pH range examined when *I* = 1.0 (data not shown). When *I* = 0.1, this UV peak does show a transition between 50 and 75°C over the entire pH range examined (Fig. 4C). This indicates that the tertiary structural change around the Phe residues could be ionic strength dependent. As previously observed for the first peak at low ionic strength (Fig. 4A), the first inflection point of the transition starts at a lower temperature at high pH (8) than that at low pH (3) (Fig. 4C), suggesting that the micro-environment around the Phe residues is less stable under higher pH conditions. The position of the second peak also undergoes blue shifts during the transition, again suggesting exposure of the residues to an environment of higher polarity. In the reduced EC5 domain, the second peak does not show shifts at most pH values upon heating. Once again, the peak shift at pH 4 appears to be very noisy due to protein precipitation (Fig. 4D).

When *I* = 1.0, the Trp peak shows a blue shift from 65°C to the final experimental temperature of 85°C (Fig. 5A). pH does not appear to influence the shifts in the UV Trp peak, suggesting that the microenvironment of the Trp residue is less sensitive to pH change than is that of the Phe residues. In contrast, the thermal transition of the Trp residue starts approximately 8°C earlier at low (Fig. 5B) than at high ionic strength (Fig. 5A). At pH 8, the thermal transition occurs approximately 3°C earlier than under other pH conditions (Fig. 5B), which is consistent with the conformational data from the Phe residues (Fig. 4). This indicates that the tertiary structure of EC5 is less stable at pH 8 than that at other pH values in low ionic strength media. Reduced EC5 displayed small transitions at pH 5–8 with a

decreasing transition temperature as the pH was increased. Similar to the previous Phe analysis, EC5 precipitation at pH 4 makes this spectrum very noisy (data not shown).

Optical Density

Turbidity measurements monitoring the optical density at 350 nm were used to investigate the gross aggregation and precipitation properties of EC5. At an ionic strength of 1.0, native EC5 does not show any evidence of gross aggregation or precipitation between pH 5 and 8 upon heating to 85°C (Fig. 6A); in contrast, aggregation of EC5 is observed at pH 3 and 4, starting around 70°C. At an ionic strength of 0.1, no significant aggregation is observed upon heating over the entire pH range examined (data not shown). In contrast, reduced EC5 forms aggregates and precipitates at pH 4 and 5 upon heating (Fig. 6B); transitions are observed around 30 and 35°C for pH 4 and 5, respectively. After the transition, extensive precipitation can be observed as the optical density begins to decrease. Significant aggregation and precipitation are not observed at other pH values.

Empirical Phase Diagrams

To better evaluate the physical and structural stability of native and reduced EC5, spectroscopic data containing both secondary and tertiary structure as well as aggregation information were combined into empirical phase diagrams using the Matlab program. At an ionic strength of 1.0, the phase diagram shows two major phases (blue and green colors) with a small transition region. The blue color state from 10 to 68°C seen over the entire pH range examined represents the stable phase of EC5 (Fig. 7A). The green region from 74 to 85°C, again seen over the entire pH range examined, represents severely structurally altered or aggregated states. The transition region between 68 and 74°C displays a molten globular behavior of EC5 in which it maintains secondary structure while tertiary structure is disrupted, *i.e.*, the thermal transitions estimated from CD spectra are approximately 6°C higher than those estimated from emission peak shifts in intrinsic fluorescence spectra. When the ionic strength is decreased to 0.1 (Fig. 7B), the physical stability of native EC5 is dramatically decreased. EC5 is stable only from 10 to 57°C. The molten globule-like states occupy a much larger region with lower temperature boundaries. Finally, the empirical phase diagram of reduced EC5 shows more dramatic and variable changes at different temperatures (Fig. 7C). Because the original spectroscopic data are very noisy, the phase diagram also becomes noisy (*i.e.*, wide color variation) and structural changes are more difficult to predict. It is clear, however, that this protein is not stable at pH 4 and 6. It appears to be partially unfolded at pH 3, 5, 7, and 8 at low temperature and severely aggregates at higher temperatures.

DISCUSSION

One of the difficulties in developing protein drugs is in finding a formulation that ensures a long shelf-life. Studies of physical stability are one of the most important steps during formulation development. This work systemically evaluates the physical stability of secondary and tertiary structures of the EC5 domain of E-cadherin as a function of ionic strength, pH, and temperature. It is clear that increasing the ionic strength increases the secondary and tertiary structure stability of EC5. The T_m of EC5 is 10°C higher at high ionic strength ($I = 1.0$) than at low ionic strength ($I = 0.1$) in a wide range of pH values. The change of ionic strength can influence the stability of proteins¹⁶⁻¹⁸ and nucleic acids,¹⁹ the activity of enzymes,²⁰ and the interaction between two proteins.²¹ The increase in ionic strength could have a stabilizing or destabilizing effect on a protein, depending on the nature of charge-charge interactions within the protein.¹⁶ The stabilization effect of the high ionic strength on EC5 is presumably due to the effective screening of the long-range charge-charge interactions on the protein surface. The EC5 domain is derived from a calcium-

binding protein E-cadherin with a high negative charge on its surface. The negative charges on EC5 repel each other at low salt concentrations, which results in the low structural stability. At high salt concentrations, these negative charges are screened by the salt and stabilize the protein structure. Because it is stabilized by high salt concentrations, EC5 can be considered a halophilic as opposed to halophobic protein.^{16,22} This effect of ionic strength on thermostability of proteins has been shown previously on cold shock protein,¹⁶ N-terminal domain of ribosomal protein L9 (NTL9),¹⁷ and nucleic acids.¹⁹

Other possible mechanisms of stabilization of proteins are specific ion binding to the protein, the increase in Hofmeister effects, and preferential protein hydration. Although it is possible, there is no evidence that EC5 is stabilized by the specific ion binding mechanism; the specific affinity binding of ion to protein is normally in low salt concentrations (0–0.2 M). The increase in ionic strength in EC5 solution could also increase the Hofmeister effect that improves the stability of the hydrophobic core or enhanced packing of the apolar interior of EC5 due to the increase in the surface tension of the solvent. This effect may provide preferential hydration of EC5 to increase its structural stability.^{23,24}

EC5 is also a good model protein for studying the effect of disulfide bonds on protein stability. The two disulfide bonds in EC5 are essential in maintaining its physical stability. The reduced form of EC5 has a T_m about 15°C lower than that of the native protein and, upon temperature increase, this reduced EC5 readily forms aggregates and precipitates. Between pH 6 and 8, EC5 does not exhibit a well-defined structure. The empirical phase diagrams (Fig. 7) clearly indicate that native EC5 is more stable than reduced EC5 over a wide range of pH and temperature. It has been previously shown that adding a disulfide bond can increase the melting temperature of proteins (*i.e.*, DHFR,^{25–27} subtilisin,^{28,29} and T4 lysozyme^{30,31}); this is presumably due to an increase in conformational rigidity and molecular packing.^{32,33} In addition, disulfide bonds have been shown to increase the overall free energy change during the unfolding process.³⁴ The effect of disulfide bonds on stabilizing proteins may also depend on temperature, *i.e.*, the effect can be more significant at higher than at lower temperature.³³ It should be noted, however, that the addition of engineered disulfide bonds does not always increase protein stability.³⁰ Furthermore, the bioactivity and folding kinetics of proteins may also be governed by the presence of disulfide bonds,^{33,35,36} but that has not been evaluated here.

Although results from the various analytical measurements used here are generally quite consistent with one another, some differences do exist. It is often quite challenging to combine the results from multiple analytical measurements to provide a global picture of the stability of proteins. Thus, the use of phase diagrams developed by Kueltzo et al.¹³ and Fan et al.^{37–39} integrates data generated by a variety of methods and makes the interpretation of the overall stability of target proteins both simpler and more rapid. Currently, empirical phase diagrams are being used for biophysical characterization^{13,37–39} as well as the basis for excipient screening for therapeutic proteins and vaccines^{13,38} among other applications. The flexibility of the empirical phase diagram approach is one of its advantages for evaluating protein stability and for formulation development. For example, the phase diagram variable axes are not limited to pH and temperature but can include other parameters such as ionic strength, excipient concentrations, agitation stress, and freeze/thaw behavior. Of course, empirical phase diagrams must be used with caution. First, the colors in the diagrams do not have any absolute meaning; they do not, for example, correspond to specific conformations of proteins. In the same diagram, if two regions are similar in color, this does not necessarily mean there are similar physical states present since similar colors can be produced by the addition of quite different vector components. It is also not valid to draw conclusions by comparing colors between two different phase diagrams. It is possible, however, to process all of the data together, thus permitting such comparisons. Secondly, a

phase diagram will tend to vary dramatically in color when the data that are used to construct the diagram are themselves very noisy. It is always necessary to directly compare the phase diagram to the data from the various methods to permit any type of mechanistic interpretation of the results.

In conclusion, this study shows that disulfide bonds have important roles in the physical stability of EC5 and contribute to the high thermal stability of EC5. Ionic strength also has a significant effect upon the stability of EC5. Finally, the use of the empirical phase diagrams enhances the interpretation of protein physical stability studies such as this.

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Figure 1.
A homology-based model of EC5.

Figure 2.

Far-UV CD spectra and the thermal stability of 20 μ M EC5 at 10°C, pH 3–8 and $I = 0.1$. CD spectra of the (A) native EC5 and (B) reduced EC5. The change in relative molar ellipticity at 230 nm of the (C) native EC5 and (D) reduced EC5.

Figure 3.

The effect of temperature on the fluorescence spectrum of 20 μ M EC5 at pH 3–8 and $I = 0.1$. Relative intrinsic fluorescence peak intensity change with temperature for the (A) native EC5 and (B) reduced EC5. Temperature-induced intrinsic fluorescence emission peak shift for the (C) native EC5 and (D) reduced EC5.

Figure 4.

The effect of temperature on the positions of peak 1 and peak 2 of the second-derivative UV absorbance spectrum of the Phe residue of 20 μM EC5 at pH 3–8 and $I = 0.1$. Temperature-dependent position of peak 1 of the (A) native EC5 and (B) reduced EC5. Temperature-dependent position of peak 2 of the (C) native EC5 and (D) reduced EC5.

Figure 5. Temperature-induced Trp peak shifts (peak 6 in second-derivative UV absorbance spectra) of 20 μ M EC5 at pH 3–8. A: Native EC5 at $I = 1.0$. B: Native EC5 at $I = 0.1$.

Figure 6. Temperature-induced optical density changes at 350 nm of 20 μ M EC5 at pH 3–8. A: Native EC5 at $I = 1.0$. B: Reduced EC5 at $I = 0.1$.

Figure 7.

Empirical phase diagrams of EC5 based upon far-UV CD, intrinsic fluorescence, and second-derivative UV absorbance spectroscopies. A: Native EC5 at $I = 1.0$. B: Native EC5 at $I = 0.1$. C: Reduced EC5 at $I = 0.1$.