The effectiveness of the first two groups is due to their size and stiffness, while the effectiveness of SO_2 is probably related to its quite large size. Finally, the difference found on this respect between alcoholic and carbonylic functions might be due to their different degrees of solvation as also revealed by the much higher water solubility of L_2. In fact, a strongly coordinated shell of water molecules exists around the OH group,[20] and this is probably responsible for its relatively high shielding ability.

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Registry No. L_1 copolymer, 79536-27-9; L_3 copolymer, 84280-01-3; M_2, 59406-68-7; L_1 SRU, 79536-61-1; L_3 SRU, 84280-00-2; divinylsulfone, 77-77-0; dimethylamine, 124-40-3.

References and Notes

(1) Morawetz, H. "Macromolecules in Solution", 2nd ed.; Wiley Interscience: New York, 1980.
(2) Barbucci, R.; Casolaro, M.; Ferruti, P.; Barone, V.; Lelj, F.; Oliva, L. *Macromolecules* **1981**, *14*, 1203 and references therein.
(3) Gramain, Ph.; Frêre, Y. *Polymer* **1980**, *21*, 921.
(4) Barbucci, R.; Barone, V.; Ferruti, P.; Oliva, L. *J. Polym. Sci., Polym. Symp.* **1981**, *69*, 49.
(5) Barbucci, R.; Ferruti, P. *Polymer* **1979**, *20*, 1061.
(6) Barbucci, R.; Ferruti, P.; Improta, C.; La Torraca, M.; Oliva, L.; Tanzi, M. C. *Polymer* **1979**, *20*, 1298.
(7) Ferruti, P.; Oliva, L.; Barbucci, R.; Tanzi, M. C. *Inorg. Chim. Acta* **1980**, *41*, 25.
(8) Ferruti, P.; Danzo, N.; Oliva, L.; Barbucci, R.; Barone, V. *J. Chem. Soc., Dalton Trans.* **1981**, 539.
(9) Barbucci, R.; Ferruti, P.; Improta, C.; Delfini, M.; Segre, A. L.; Conti, F. *Polymer* **1978**, *19*, 1329.
(10) Andreani, F.; Angeloni, A. S.; Angiolini, L.; Bizzarri, P. C.; Della Casa, C.; Fini, A.; Ghedini, N.; Tramontini, M.; Ferruti, P. *J. Polym. Sci., Polym. Lett. Ed.* **1981**, *19*, 443.
(11) Angeloni, A. S.; Tramontini, M.; Ferruti, P., submitted for publication.
(12) Micheloni, M.; Sabatini, A.; Vacca, A. *Inorg. Chim. Acta* **1977**, *25*, 41.
(13) Sabatini, A.; Vacca, A.; Gans, P. *Talanta* **1974**, *21*, 53.
(14) Felty, W. J. *J. Chem. Educ.* **1978**, *55*, 577.
(15) Hamilton, W. C. "Statistics in Physical Sciences"; The Roland Press: New York, 1964.
(16) Scarborough, B. "Numerical Mathematical Analysis"; J. Hopkins Press: Baltimore, MD, 1930, 187.
(17) Barbucci, R.; Ferruti, P.; Micheloni, M.; Delfini, M.; Segre, A. L.; Conti, F. *Polymer* **1980**, *21*, 81.
(18) Lewis, E. A.; Barkley, J.; St. Pierre, T. *Macromolecules* **1981**, *14*, 546.
(19) Rinaldi, P. L.; Yu, C.; Levj, G. C. *Macromolecules* **1981**, *14*, 551.
(20) Meot-Ner, M.; Hamlet, P.; Hunter, P. E.; Field, F. H. *J. Am. Chem. Soc.* **1980**, *21*, 6393. Barbucci, R. *Inorg. Chim. Acta* **1975**, *12*, 113.

α-Helix-to-Random-Coil Transition of Two-Chain, Coiled Coils. Theory and Experiments for Thermal Denaturation of α-Tropomyosin at Acidic pH

Marilyn Emerson Holtzer, Alfred Holtzer,* and Jeffrey Skolnick

Department of Chemistry, Washington University, St. Louis, Missouri 63130.
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ABSTRACT: New data are presented for the α-helix content of non-cross-linked and cross-linked α-tropomyosin at acidic pH as a function of temperature (20–80 °C) over a 1000-fold range of protein concentration. Experimental thermal denaturation curves at acidic pH are independent of concentration for both non-cross-linked and cross-linked samples, unlike the case of neutral pH, where it was previously shown that the non-cross-linked protein data are concentration dependent. The α helix in the non-cross-linked case is more stable than in the cross-linked case at low pH and both are considerably more stable than at near-neutral pH. The realized theory developed earlier is applied to these new data. The theory shows that even noninteracting helices are considerably more stable at acidic pH because of substantial increases in the helix propagation parameter $s(T)$ for aspartic and glutamic acids over those for aspartate and glutamate, respectively. The theory for interacting helices fits the data well with an interaction parameter $w(T)$ that is only slightly larger than that required to fit the data at neutral pH. Thus, most of the enhanced tropomyosin helix stability in acid has its origin in enhanced short-range interactions [$s(T)$] for its acidic residues. The insensitivity of $w(T)$ to pH implies that the salt linkages, which cannot be present in acid, do not contribute appreciably to the helix–helix interaction even when present at neutral pH. The theory also reveals that dissociation to monomers is entirely negligible at low pH over the full range of conditions employed, explaining the lack of dependence of the observed helix content on protein concentration. The reduced helix content of the actual cross-linked dimers, as compared with both theory and experiment for non-cross-linked dimers, confirms an earlier conclusion (drawn less directly from the data on the partially dissociated system at neutral pH) that the actual disulfide cross-link has a locally adverse effect on the helix-stabilizing interactions.

I. Introduction

In preceding papers in this series a statistical mechanical theory is described that treats the α-helix-to-random-coil transition of two-chain, coiled coils[1] and the theory is applied to extant experiments on a synthetic model polypeptide and to new experiments on α-tropomyosin.[2,3] The theory is implemented by use of the extensive compilation by Scheraga et al. of the helix initiation (σ) and propagation [$s(T)$] parameters that characterize the short-range interactions for each type of amino acid residue.[4] Fitting the theory to experimental data for the fraction helix (Φ_h) vs. temperature determines a temperature-dependent parameter $w(T)$ that characterizes the helix–helix interaction in the coiled coil. In this manner, the theory makes possible a partial dissection into its constituent parts of the free energy responsible for the overall coiled coil stability.

Tropomyosin is an important muscle protein whose native molecular structure has for some time been known to be that of a double α-helical, coiled coil.[4] This molecular architecture is certainly a result of the remarkable pseu-

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dorepeating heptet of residues that characterizes the amino acid sequence.[4] The thermal denaturation of the protein near neutral pH has been thoroughly investigated and, through use of the statistical mechanical theory, at least partly understood.[1-4]

Long before any appropriate theory existed and long before the tropomyosin sequence was known, it had been observed that the α helix in tropomyosin (and in paramyosin, also an α-helical coiled coil) is more stable at pH 2 than at near-neutral pH.[5-8] At first sight this is a puzzling result. The most obvious effect that attends the reduction in pH from 7 to 2 is the discharging of the carboxyls. The most obvious physical effects that would then ensue are (1) a substantial increase in the net charge of the molecule and (2) destruction of the interhelix salt links that (along with hydrophobic interactions) allegedly constitute the interhelix stabilizing force. Both effects ought to make the structure *less* stable at low pH, contra experiment. Although various proposals have been made,[5-8] no clear-cut explanation of this seeming paradox has appeared.

Because the theory is designed to separate the various stabilizing factors, we felt that application of it to the problem of tropomyosin stability at low pH might shed light on these apparent contradictions. For this reason, we reinvestigated the circular dichroism of α-tropomyosin at pH 2 as a function of temperature and protein concentration and fitted the data to the theory as in the previous work.[1-3] We report the results herein along with relevant comparisons with the state of α-tropomyosin near neutral pH.

II. Methods

Virtually all the experimental techniques are identical with those described previously.[3] In the present instance, measurements were made on non-cross-linked α-tropomyosin in $(NaCl)_{590}(HCl)_{10}(DTT)_{0.5}(2.0)$; for the cross-linked material, the DTT was omitted.[9] Particular care was taken to ensure reversibility of the thermal denaturation curves because of the possibility of chain scission or of glutamine or asparagine deamidation in acid at the higher temperatures. No difficulties that might be reasonably ascribed to such effects were observed. Since $s(T)$ values are not available above 70 °C, measurements were only taken up to 80 °C.

Fraction helix was calculated from eq 6 of ref 3, which requires an estimate of I, the average number of helical regions in a molecule. Previously we employed the estimate $I = 2$ throughout. This time, $I = 1$ was used to initiate a calculation by successive approximations, as described (but not implemented) previously.[3] Final values of I were near 2 and for the cases under consideration the present method does not differ materially from the earlier one.

The theory was utilized precisely as before. The input σ and $s(T)$ parameters were the same, except, of course, for the aspartic and glutamic acid residues, which differ in measured short-range interactions from aspartate and glutamate, respectively.[10,11] To obtain the values appropriate to low pH, we must alter Table I of our earlier work.[2] The appropriate substitutions are as follows. For Asp, $\sigma = 0.021$, $B_0 = -7.186$, $B_1 = 3724.8$, and $B_2 = -496\,890$; for Glu, $\sigma = 0.010$, $B_0 = -1.6047$, $B_1 = 557.82$, and $B_2 = 0.0$. Using these and the other values with the theory, we obtained the dependence of the helix–helix interaction parameter $w(T,2)$ appropriate to α-tropomyosin at temperature T and pH 2.0 by fitting to a function of the form of eq 7 of ref 3, just as before. The result was then used in the theory as usual to calculate overall fraction helix, fraction of dimer, dimer helix content, etc.

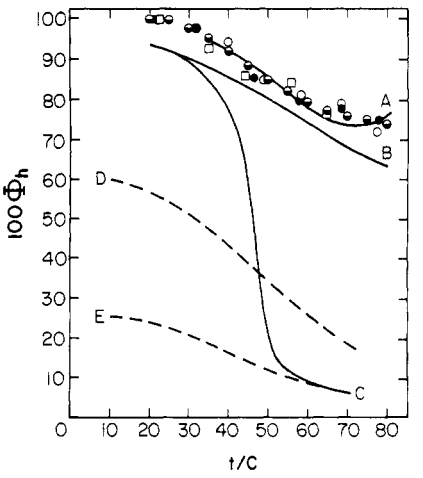

Figure 1. Percent helix vs. temperature for non-cross-linked α-tropomyosin. Experimental points are acid solutions at various protein concentrations in $(NaCl)_{590}(HCl)_{10}(DTT)_{0.5}(2.0)$. Open squares, 0.0051 mg·mL^{-1}; open circles, 0.0058 mg·mL^{-1}; half-filled circles, 0.12 mg·mL^{-1}; filled circles, 5.2 mg·mL^{-1}. Solid curve A, theory using all parameters applicable to acid solutions, i.e., $\Phi_h[\sigma(2),s(T,2),w(T,2)]$. Solid curve B, theory for acidic short-range interactions with neutral pH interhelix interactions, i.e., $\Phi_h[\sigma(2),s(T,2),w(T,7)]$. Solid curve C, theory for pH 7, i.e., $\Phi_h[\sigma(7),s(T,7),w(T,7)]$, at 0.104 mg·mL^{-1} protein concentration. Dashed curve D, theory for noninteracting helices at pH 2, i.e., $\Phi_h[\sigma(2),s(T,2),w = 1]$. Dashed curve E, theory for noninteracting helices at pH 7, i.e., $\Phi_h[\sigma(7),s(T,7),w = 1]$.

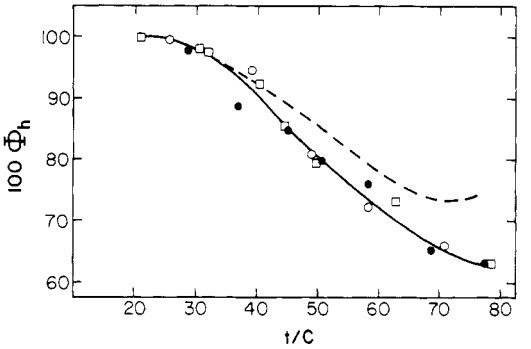

Figure 2. Percent helix vs. temperature for cross-linked α-tropomyosin. Experimental points are acid solutions at various protein concentrations in $(NaCl)_{590}(HCl)_{10}(2.0)$. Open circles, 0.0050 mg·mL^{-1}; open squares, 0.011 mg·mL^{-1}; filled circles, 5.1 mg·mL^{-1}. Solid curve, spline curve through data. Dashed curve, theory using all parameters applicable to acid solutions, i.e., $\Phi_h[\sigma(2),s(T,2),w(T,2)]$, same as curve A of Figure 1.

III. Experimental Results

The experimental points for non-cross-linked α-tropomyosin at pH 2.0 are shown on Figure 1 as percent helix vs. temperature over a 1000-fold range of protein concentration. The results are in clear contrast with those shown earlier for near-neutral pH (solid curve C on Figure 1) in that at low pH the molecule is, as already known,[5-8] much more stable at pH 2, being over 70% helix even at the highest temperature (70–80 °C) at which the theory can be realized. Furthermore, the dependence on protein concentration observed earlier at neutral pH[3] is not evident in acid solutions. Points for all concentrations at pH 2 are seen in Figure 1 to fall on essentially the same empirical curve (not shown). The significance of the other curves on Figure 1 will be discussed below.

The experimental points for cross-linked α-tropomyosin at pH 2.0 are shown on Figure 2. These demonstrate that the cross-linked material is slightly less stable than the non-cross-linked case at pH 2, but more stable than the

cross-linked case at pH 7, and also show no dependence on protein concentration.

IV. Theoretical Results and Discussion

Noninteracting Helices. As previously emphasized, it is always enlightening to establish, as a base line, the behavior of dimers containing noninteracting helices, i.e., helices with $w = 1$. Since, given the amino acid sequence, the theory provides various quantities as a function of the array of σ, s, and w values, each of which may be dependent upon temperature, pH, or both, some convenient notation is desirable. We will employ, then, $\Phi_h[\sigma(\text{pH}),s(T,\text{pH}),w(T,\text{pH})]$ to mean a set of values of overall fraction helix calculated from theory by employing the array of σ, s, and w values characteristic of the indicated pH and temperature. For noninteracting helices at pH 2 we thus have the theoretical denaturation curve $\Phi_h[\sigma(2),s(T,2),w = 1]$. This curve is shown as dashed curve D on Figure 1 along with the corresponding curve (dashed curve E) for noninteracting helices near neutral pH, i.e., $\Phi_h[\sigma(7),s(T,7),w = 1]$.

Comparison of curves D and E reveals a most striking difference between tropomyosin at acidic and at neutral pH and goes a long way toward explaining the pronounced increase in stability in acid. Whereas single (i.e., noninteracting) chains form rather unstable helices at pH 7, being only ~25% helical at the lowest attainable temperatures and an almost negligible 7% helical at 70 °C, such chains at pH 2 would vary from almost 60% helical to almost 20% over the same temperature range. This marked stabilization is entirely due to the increase in $s(T)$ for aspartic and glutamic acids over the corresponding values for aspartate and glutamate and to the sizable number of such residues in the molecule. Thus, in good part, the augmented stability of tropomyosin α helices at pH 2 is a result of enhanced short-range interactions, in particular the improved value of the helix propagation parameter, of its acidic residues.

Since most proteins contain substantial numbers of such residues, one would not expect this effect to be confined to tropomyosin. One might expect a similar enhancement for other proteins in solutions of sodium dodecyl sulfate, for example, wherein the native structure may be irrelevant, perhaps only α helix and randomly coiled secondary structures exist, and the present type of theory therefore applies.[12] Such enhancement of helix content in acidic dodecyl sulfate solutions over that seen in neutral dodecyl sulfate solutions has in fact been observed but ascribed to another cause, i.e., the loss (at low pH) of charge–charge repulsions from local clusters of acidic groups.[13] We would argue first, that the increase in *net* charge that accompanies acidification probably produces a net increase in electrostatic free energy of the helix rather than a decrease; second, that the persistence of the effect even at the relatively high ionic strengths reported here makes it unlikely that any charge–charge interactions are crucial in its genesis; and third, that the use of the same Chou–Fasman parameters as a measure of "helix-forming tendency" at both acidic and neutral pH can mislead one to think that these tendencies are independent of pH.[12] In fact, as shown here, examination of the $s(T,\text{pH})$ values shows that these tendencies tip the balance strongly in favor of helix at low pH. It remains to be seen whether this remains true in the presence of sodium dodecyl sulfate. Present evidence favors the view that, except for cationic residues, $s(T)$ values are insensitive to the presence of that detergent.[12] If so, then acidification of dodecyl sulfate containing protein solutions should considerably enhance the helix content, because the effect is rather large in proteins with appreciable aspartic and glutamic content.

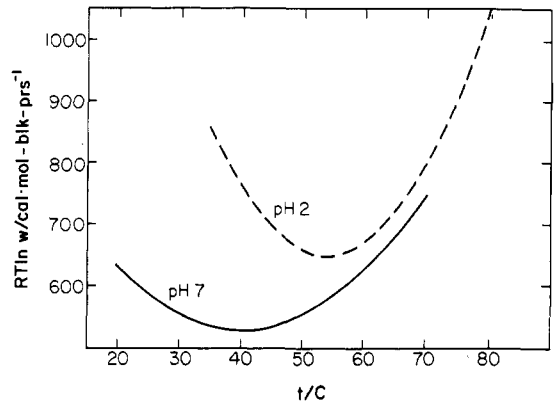

Figure 3. Helix–helix interaction (RT ln w/(cal·(mol of block pairs)$^{-1}$) vs. temperature at acidic and at neutral pH.

Non-Cross-Linked α-Tropomyosin. The data of Figure 1, used exactly as before to obtain $w(T,2)$, provide results qualitatively too similar to those obtained previously to bear repeating. The resulting computer-fit curve for RT ln $w(T,2)$ is given by eq 7 of ref 3 with the numerical values A_0 = 63 227.5956, A_1 = −382.424 561, A_2 = 0.585 725 092, and B = −0.082 958 899 2. The curves for pH 2 and pH 7 are compared on Figure 3.

When the alogrithm for RT ln $w(T,2)$ is employed in the theory, the calculated values of percent helix provide the solid curve A shown on Figure 1. Plainly, the theory agrees with the measurements within experimental error. Furthermore, the theory explains the lack of dependence of the observed helix content on protein concentration. With the array of values of $\sigma(2)$ and $s(T,2)$ employed and the $w(T,2)$ shown on Figure 3, the theory provides not only the $\Phi_h[\sigma(2),s(T,2),w(T,2)]$ shown on Figure 1 but also the values of the relative populations of two-chain and single-chain molecules. The results show, for the entire investigated range of temperature and protein concentration, an entirely negligible population of single chains. Thus, although the chains are not cross-linked in these experiments, they remain quantitatively associated, and the system shows no effects of mass action.

Although Figure 3 shows what appears to be a substantial difference in interhelix interactions between acidic and neutral pH, these differences are, in fact, barely detectable. At the high helix content always observed at pH 2, the helix content is a very insensitive function of $w(T,\text{pH})$. Consequently, the value of w obtained depends very markedly on the precise values of fraction helix used to obtain the fit. Thus, any small error in Φ_h translates into a rather large error in w. This can most easily be seen by employing in the theory the pH 7 values for the helix–helix interaction free energy along with the proper pH 2 short-range interactions. The resulting calculated values of $\Phi_h[\sigma(2),s(T,2),w(T,7)]$ are shown as the solid curve B on Figure 1, which is seen to be displaced downward from the experimental values by about a mere 5 percentage points in helix content. It is impossible at present to be sure that such a small difference in helix content is in fact significant. A small pH dependence of the mean residue ellipticity for the intact helix or random coil might easily produce enough of a shift in the experimental points to account for the observed difference.

We thus conclude that the mean helix–helix interaction is certainly no smaller at pH 2 than at neutral pH and may be somewhat larger. In either event, that interaction is strong enough so that, acting in concert with the augmented s at pH 2, it effectively bars dissociation into single

chains and produces a strongly augmented helix content. The contrast with the curve calculated for an intermediate protein concentration using all parameters at pH 7, i.e., $\Phi_h[\sigma(7),s(T,7)w(T,7)]$, shown as solid curve C on Figure 1, is striking. At pH 7, the marked decrease in favorable short-range interactions (along with relatively little change in w) results in dissociation and attendant drastic drop in helix content in the accessible temperature range.

The conclusion above that the helix–helix interaction is, if changed at all, somewhat stronger at low pH is at first sight surprising. As previously noted,[2] this interaction is a composite of several molecular effects. Most prominent among them are, perhaps, hydrophobic and the salt-linkage interactions.[4] The hydrophobic interactions are generally believed to the pH independent. Since the salt linkages are removed at pH 2, one would have expected the residual helix–helix interaction to decrease at low pH. The constancy (or slight increase) of that interaction would point to the tentative conclusion that, at the high ionic strengths employed in these experiments, the salt linkages at pH 7 do not contribute very much to the overall helix–helix interaction. A more definitive conclusion will have to wait upon more thorough dissection of the interhelix interaction.

It perhaps has not escaped the reader's notice that theoretical curve A in Figure 1, i.e., $\Phi_h[\sigma(2),s(T,2),w(T,2)]$, displays a shallow minimum near 72 °C. This occurs as a result of the sharply increasing $w(T,2)$ in this temperature region, which overwhelms the decreasing $s(T,2)$. Unfortunately, although the possibility of an increase in helix content with temperature is intriguing, neither experiment nor theory can be relied upon at present for a definitive answer. The present experimental data (Figure 1) show a degree of scatter that makes them not inconsistent with such a minimum, but certainly not demonstrative of it. Further experimentation may be fruitful, but the region above 80 °C at pH 2 is fraught with technical difficulties. The theory is also rather helpless in this region. Not only does the persisting high helix content make the determination of w uncertain, but the measurements of s(T) usually do not extend above 70 °C, so even the theoretical fits in the 70–80 °C region require some extrapolation (by algorithm) of the actual data.

Cross-Linked α-Tropomyosin. We next turn our attention to the helix content of the cross-linked material at low pH. On Figure 2 the solid curve is a spline curve drawn to represent the data. The calculated curve $\Phi_h[\sigma(2),s(T,2),w(T,2)]$, i.e., solid curve A of Figure 1, is shown as the dashed curve on Figure 2. Since no dissociation is predicted by theory in this range, the two curves (each of which well represents the respective experiments) show rather directly the comparison between the helix content of a non-cross-linked dimer and of a cross-linked dimer. It will be recalled that such a direct comparison is impossible for the pH 7 case, since dissociation at pH 7 complicates the non-cross-linked case, requiring us to deduce from the experiments the behavior of the non-cross-linked dimers.[3] As Figure 2 shows, the present, more direct, experimental comparison confirms our previous conclusion that the presence of an actual physical disulfide cross-link reduces the helix stability over a dimer with no such real cross-link. Whether this occurs because the cross-link alters the short-range (σ,s) interactions or the long-range (w) interactions, or both, in its vicinity is, at this stage, impossible to tell.

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References and Notes

(1) Skolnick, J.; Holtzer, A. *Macromolecules* **1982**, *15*, 303–314.
(2) Skolnick, J.; Holtzer, A. *Macromolecules* **1982**, *15*, 812–821.
(3) Holtzer, M. E.; Holtzer, A.; Skolnick, J. *Macromolecules* **1983**, *16*, 173.
(4) For a full list of references, see ref 3.
(5) Noelken, M. Ph.D. Thesis, Washington University, 1962.
(6) Noelken, M.; Holtzer, A. "Biochemistry of Muscle Contraction"; Little, Brown and Co.: Boston, 1964; pp 374–378.
(7) Woods, E. F. *Biochemistry* **1969**, *8*, 4336–4344.
(8) Woods, E. F. *Aust. J. Biol. Sci.* **1976**, *29*, 405–418.
(9) We describe complex aqueous solvent media by giving the chemical formula of each solute in parentheses with its millimolarity as subscript, followed by the pH in parentheses. Other abbreviations: DTT, dithiothreitol; CD, circular dichroism.
(10) Maxfield, F.; Alter, J.; Taylor, G.; Scheraga, H. *Macromolecules* **1975**, *8*, 479.
(11) Kobayashi, Y.; Cardinaux, F.; Zweifel, B.; Scheraga, H. *Macromolecules* **1977**, *10*, 1271.
(12) Mattice, W.; Riser, J.; Clark, D. *Biochemistry* **1976**, *15*, 4264–4272.
(13) Wu, C.-S.; Ikeda, K.; Yang, J. T. *Biochemistry* **1981**, *20*, 566–570.