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Stability of the folded-chain β-structure of a homopolypeptide based on time-resolved potentiometric titrations

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The standard free energy change of the unimolecular conversion between the folded-chain β-structure and random coil of uncharged poly(*S*-carboxymethyl-L-cysteine) was evaluated from the potentiometric titration curves extrapolated to zero time to reduce the effect of aggregation which occurred at slower rates than chain folding for most degrees of ionization. To reduce the remaining contribution from the aggregation, the results obtained at finite concentrations were further extrapolated to zero polymer concentration. A value of $-(400 \pm 100)$ cal/mol was obtained for a sample of chain length 630. From the titration curves at the aggregation equilibrium, the total free energy change of the whole solution associated with the same conversion was determined. The contribution made by aggregation to the stability was determined from the difference between these two quantities, which turned out to be comparable with that from the unimolecular conversion.

1. Introduction

Two important secondary structures in proteins are the α-helix and the β-structure. Since the problems involved in studying secondary structures in proteins are innumerable, resort has been made to the studies on model polypeptides. Helix-coil conversion of model polypeptides has been developed both experimentally and theoretically. Values of the parameters describing the conversion have been determined for 20 amino acids by means of the host-guest technique [1]. On the other hand, little progress has been made regarding the β-structure. This is the result of many experimental studies having been performed with poorly defined systems in the sense that β-structures in a given solution consist of both extended and folded chains. Results obtained on these solutions cannot be subjected to theoretical analyses.

In studies on folded-chain β-structures, it is not easy to achieve the desired unimolecular conversion, since aggregation of folded-chain β-structure as well as of extended chains frequently occurs even over moderately dilute concentration ranges (approx. 1×10^{-3} M or 0.1 g/l) [2–4]. In the case of poly(L-tyrosine), potentiometric titrations can be carried out at very low concentrations, since the degree of ionization can be spectrophotometrically determined with the aid of the side chain chromophore. Hence, the standard free energy change ΔG^0 has been successfully determined [5,6]. In the case of poly(L-lysine); titrations were carried out at concentrations $C_p = (1–10) \times 10^{-3}$ M, where aggregation occurred [7,8]. The correlation between formation of the β-structure and aggregation was rather extensively examined in the case of poly(*S*-carboxymethyl-L-cysteine (poly[Cys-(CH_2COOH)]) [4]. According to the result, folded-chain β-structure without aggregation is

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shown to exist at concentrations below about 1×10^{-4} M for long chains unless the charge densities of the β-structure are low [4,9]. It appears to be difficult to carry out the titrations at such low concentrations as those required for the unimolecular conversion in terms of the routine procedure which usually works at concentrations above 1×10^{-3} M. There is a way, however, to reconcile these two apparently conflicting situations, if we take note of the different relaxation times for the two processes; unimolecular conversion and aggregation. Recently, we have found that folding of homopolypeptide chains into the β-structure occurs rather rapidly when the charge density is low [10]. On the other hand, aggregation of polypeptide chains is known to occur very slowly, although its time range is diverse depending on the conditions [10,11]. It is expected that under various conditions formation of the β-structure proceeds through the folding of chains followed by slow aggregation.

In the present study, we have carried out the potentiometric titration of poly[Cys(CH_2COOH)] at low concentrations (mostly below 1×10^{-3} M) in order to reduce the effect of aggregation as much as possible. The observed time course of pH after the addition of acid is extrapolated to zero time in order to reduce further the effect of aggregation. With the aid of such time-resolved titration curves at low concentrations, we are able to evaluate, although only approximately, ΔG^0 and the contribution made by aggregation to the stability of the β-structure.

2. Experimental

The weight-average molecular weights (degrees of polymerization DP_w) of the two samples were 10.1×10^4 (630) and 5.8×10^4 (360).

Potentiometric titrations were carried out under an N_2 atmosphere in two ways, designated as methods C and D in the previous studies [11,12]. The volume of the solutions was 20.0 and 10.0×10^{-3} dm^3 for method C and D, respectively. Delivery of HCl or NaOH solutions was effected with an electric buret (Schott-Gerate, T100) with cylinders of 1.000×10^{-3} dm^3. Measurements of pH were carried out with a Beckman Φ71 pH meter using a combination electrode (Horiba no. 6327-10c). Calibrations of the electrode were made with NBS standard buffers. The temperature was maintained at 25.0 ± 0.1 °C. In method C, the routine procedure for titration, a solution was titrated continuously by adding successively either HCl or NaOH solutions to cover the entire range of ionization. In method D, a number of solutions of the same composition were prepared and incubated. Time courses of pH at a given degree of neutralization were obtained with method D.

Circular dichroism (CD) of the solutions was measured with a Jasco J40A spectropolarimeter using cells of 10 and 5 mm path lengths at 25 ± 0.5 °C.

The NaCl concentration was kept constant at 5.0×10^{-2} M throughout the present study. The polypeptide concentration C_p, expressed in residue molarity, was below 1×10^{-3} M in most cases.

3. Results

In this paper, forward and backward titrations are defined as those accompanying the protonation to carboxylate groups and the dissociation of carboxyl groups, respectively. The present convention is the reverse of that used previously [11,12]. The effect of polypeptide concentration C_p was examined in terms of method C using two samples, $DP_w = 630$ and 360, as shown in fig. 1A and B, respectively. The backward titration curves, denoted by open and filled circles, were independent of C_p. In contrast, the curves for the forward titration, represented by dotted and solid curves, depend significantly on concentration and do not coincide with those of the backward titration in the conformational conversion region. Hysteresis was thus observed for this region and the area bounded by the loops increased as the concentration difference increased. In the regions where either conformation was dominant, the titration curves were reversible and independent of C_p. In fig. 1, dashed lines labelled a represent the titration curves of random coils.

In fig. 2, the two different titration procedures (methods C and D) are compared on the sample

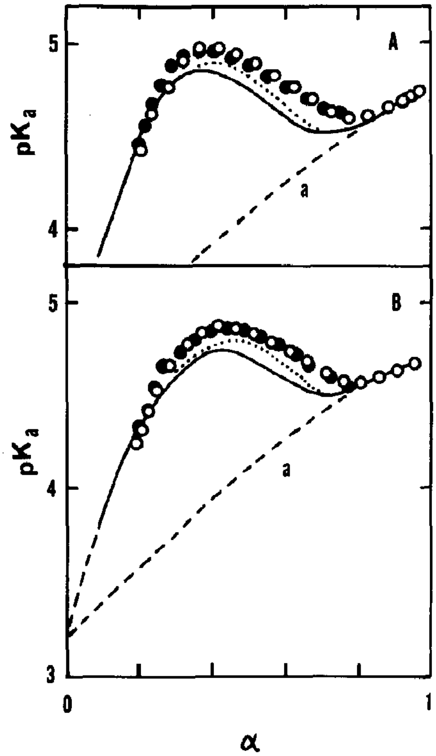

Fig. 1. Titration curves ($pK_a = pH - \log(\alpha/1-\alpha)$) of the samples with $DP_w = 630$ (A) and $DP_w = 360$ (B) in 50 mM NaCl solutions obtained with method C. (A) $C_p = 4.67 \times 10^{-4}$ M (● and solid curve) and $C_p = 1.87 \times 10^{-3}$ M (○ and dotted curve), (B) $C_p = 9.73 \times 10^{-4}$ M (● and solid curve) and $C_p = 2.12 \times 10^{-3}$ M (○ and dotted curve). Forward (backward) titrations are denoted by curves (circles). Dashed curves labelled a represent the titration curve of random coils.

with $DP_w = 630$. Reversible results were obtained with method D (circles) and coincided with the data on the backward titration obtained with method C (solid curve). Solutions for the backward titrations in method D were prepared by adding NaOH solution to the solution represented by an open square in fig. 2. The same results as shown in fig. 2 were obtained with the other sample ($DP_w = 360$).

In the previous studies [11,12], efforts were made to obtain reversible equilibrium titration curves and hence method D was recommended. In contrast, in the present study titration curves are required which correspond to solutions containing the lowest possible amount of aggregates. Consequently, forward titrations with method C have been employed throughout the following. All the results shown in figs. 1 and 2 indicate that the cause of the hysteresis loop, the concentration dependence and the difference between the two methods, is merely the time course followed in attaining aggregation equilibrium. As the amount and/or extent of aggregation increases, $pK_a = pH - \log \alpha/(1-\alpha)$ increases. The time courses observed for the pH and CD (see later) are ascribed mainly to aggregations among folded-chain β-structures and among extended chains, as well as mixed aggregates consisting of both. These aggregations proceed rather slowly compared with the folding process of isolated chains, except for the case of high charge densities. Their contributions can be reduced if the titration curves are extrapolated to zero time.

The time courses of the pH of the sample with $DP_w = 630$ at $C_p = 6.64 \times 10^{-4}$ and 2.24×10^{-4} M are illustrated for various degrees of ionization in fig. 3A and B, respectively. The corresponding time courses of $[\theta]_{205}$ are depicted in fig. 4A and B. Changes in pH occurred seemingly in two steps except for the initial drop, which was assumed to

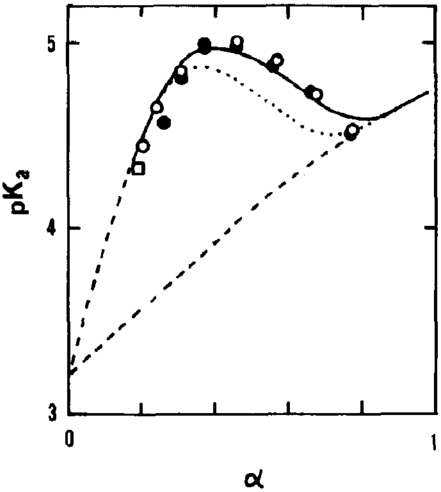

Fig. 2. Comparison of titration curves ($pK_a = pH - \log(\alpha/1-\alpha)$) obtained in terms of methods C and D. $C_p = 4.68 \times 10^{-4}$ M, $DP_w = 630$. Method D, forward (○) and backward (●); method C, forward (dotted curve) and backward (solid curve). Dashed lines represent the extrapolated branches of the β-limb and coil-limb. A square represents the solution from which backward titration in method D was carried out.

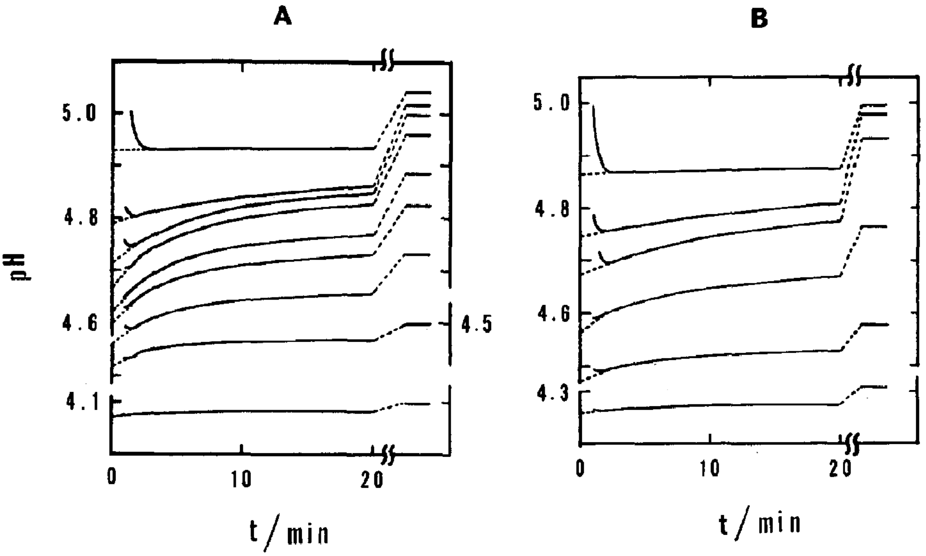

Fig. 3. Time course of pH after the addition of HCl for the sample with $DP_w = 630$ in 50 mM NaCl solutions. C_p: 6.63×10^{-4} M (A) and 2.44×10^{-4} M (B). Degrees of ionization (from top to bottom): (A) 0.73, 0.68, 0.63, 0.58, 0.47, 0.42, 0.37, 0.30, 0.23; (B) 0.63, 0.61, 0.53, 0.38, 0.32, 0.27. Bars on the right indicate the stationary values obtained 1 day after the addition of HCl.

be not related to the process under study. The faster of the two steps took place within 10–30 min, the slow one reaching completion after about 10–20 h. Approximately constant readings of the pH were obtained 10–30 min after the addition of HCl. These values were taken as the forward titration data obtained with method C. The fast step was observed only in the central part of the conversion region whereas only the slow step was evident at high and low pH values as indicated in fig. 3A and B. At low pH, both chain folding and aggregation took place very rapidly, while at high pH folding of isolated chains scarcely occurred. The time course of $[\theta]_{205}$ corresponded well to that followed by pH. The extent of the change in pH or $[\theta]_{205}$ was large in the conversion region but became small beyond the limits of this range. Results similar to those shown in fig. 3A and B were also obtained on the other sample ($DP_w = 360$).

As shown in figs. 3 and 4, the values of pH and $[\theta]_{205}$ were extrapolated to zero time ($t = 0$) and were assumed to represent those of the folded-chain β-structure accompanied by the minimum degree of aggregation.

In figs. 5 and 6, correspondence between pH and $[\theta]_{205}$ is illustrated for the two samples for values extrapolated to $t = 0$ as well as for equilibrium values. For the sample with $DP_w = 630$ at aggregation equilibrium, conversion between random coils and the β-structure occurred at an approximately constant pH (5.00), as demonstrated by the dashed curve in fig. 5A. This behavior is consistent with the previous description of the conversion in terms of microscopic phase separation [4]. On the other hand, the maximum pK_a in the conversion region is greatly reduced for the approximate unimolecular conversion at $t = 0$ (solid curve in fig. 5A). The difference between the two titration curves is least for the sample with $DP_w = 360$. The difference in amounts of β-structure between both curves ($t = 0$ and $t = \infty$) represents the contribution from various aggregations. Aggregation of the folded-chain β-structure can potentially increase the amount of β-structure since it shifts the unimolecular coil-β-structure equilibrium.

In fig. 7, titration curves of the sample with $DP_w = 630$ corresponding to $t = 0$ and $t = \infty$ (practically 1 day) are shown for different polymer concentrations. The area A, bounded by the actual titration curve and the hypothetical plot

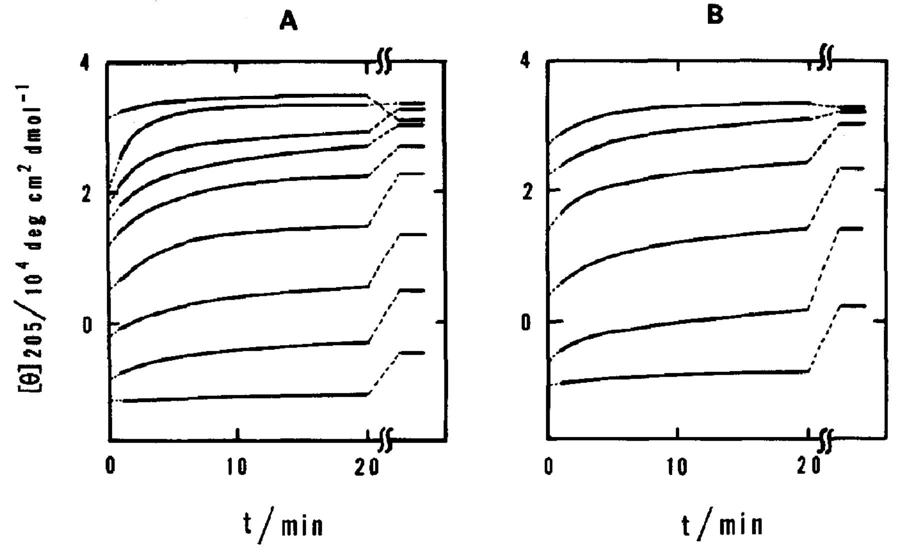

Fig. 4. Time course of $[\theta]_{205}$ after the HCl addition for the sample with $DP_w = 630$ in 50 mM NaCl solutions. C_p: 6.63×10^{-4} M (A) and 2.44×10^{-4} M (B). Degrees of ionization (from bottom to top): (A) 0.73, 0.68, 0.63, 0.58, 0.47, 0.42, 0.37, 0.30, 0.23; (B) 0.63, 0.61, 0.53, 0.38, 0.32, 0.27. Bars on the right indicate the stationary values obtained 1 day after the addition of HCl.

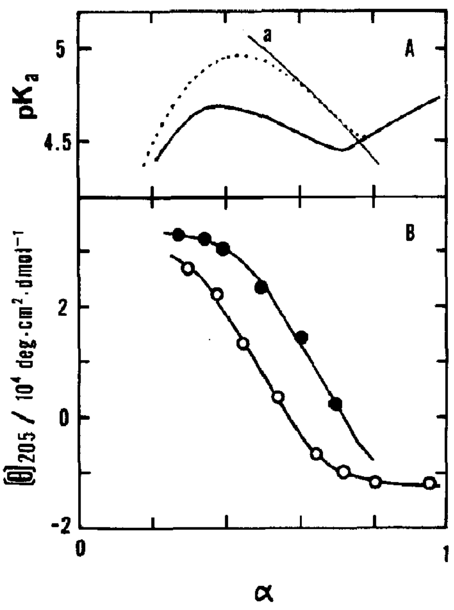

Fig. 5. Coil-β-structure conversion in two different time domains. $DP_w = 630$, $C_p = 2.44\times10^{-4}$ M. (A) Titration curves ($pK_a = pH - \log(\alpha/1-\alpha)$): solid and dotted curves correspond to the data at $t = 0$ and $t = \infty$ (1 day), respectively. The thin solid trace α represents a line of constant pH (5.00). (B) Dependence of $[\theta]_{205}$ on the degree of ionization α. Open (filled) circles represent the data at $t = 0$ ($t = \infty$).

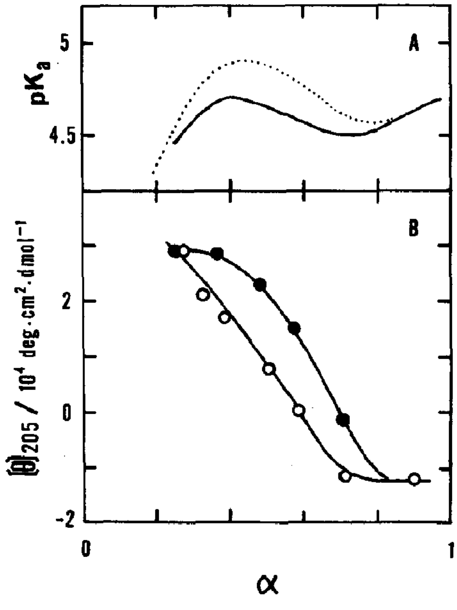

Fig. 6. Coil-β-structure conversion in two different time domains. $DP_w = 360$, $C_p = 5.31\times10^{-4}$ M. (A) Titration curves ($pK_a = pH - \log(\alpha/1-\alpha)$): solid and dotted curves correspond to the data at $t = 0$ and $t = \infty$ (1 day), respectively. (B) Dependence of $[\theta]_{205}$ on the degree of ionization α. Open (filled) circles represent the data at $t = 0$ ($t = \infty$).

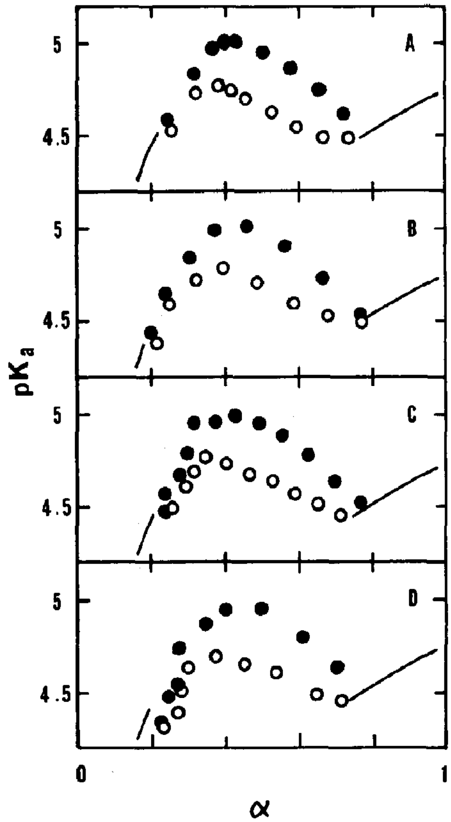

Fig. 7. Concentration dependence of the titration curves ($pK_a = pH - \log(\alpha/1-\alpha)$) of the sample with $DP_w = 630$ in 50 mM NaCl solutions. C_p (10^{-4} M): (A) 6.63, (B) 4.69, (C) 3.50, (D) 2.44. Open and filled circles represent the data at $t = 0$ and $t = \infty$ (1 day), respectively.

(labelled a in fig. 1) corresponding to random coils for the entire range of α, has been related to ΔG^0 the standard free energy change of uncharged polymer between two discrete conformations [13–16], for the case in which aggregation is absent. We tentatively assume that the area, A, from the titration curve ($t = 0$) provides good estimate of ΔG^0 according to the relation

$$-\Delta G^0 = 2.303RTA. \tag{1}$$

The area obtained from the reversible titration curve ($t = \infty$) is related [17,18] to the free energy change of the whole solution ΔG, divided by the number of moles of residues present, between the actual solution containing aggregates of uncharged β-structure and the hypothetical solutions containing uncharged random coils. In fig. 8, values of ΔG or ΔG^0 are plotted as functions of C_p for two samples of different molecular weights. For the sample with $DP_w = 630$, one observes that $-\Delta G^0$ at two high concentrations ($5-7 \times 10^{-4}$ M) is similar but diminishes as C_p decreases. This suggests that the procedure for extrapolating the titration curves to $t = 0$ was unable to remove the perturbation due to aggregation. In other words, the time resolution in the present study was not good enough. Perturbation of aggregation decreases with reduction in C_p. Hence, extrapolation of the results shown in fig. 8 to zero concentration ($C_p = 0$) is expected to give a better estimate of ΔG^0. The extrapolated value was found to be $-(400 \pm 50)$ cal/mol, as seen from the figure. This value is consistent with the predicted range (less negative than -500 cal/mol) in the previous study [4]. Lower values of $-\Delta G^0$ were obtained for the sample with $DP_w = 360$, which were however considered to include the contribution from aggregation, since extrapolation to $t = 0$ could not be carried out for this sample. The result indicates

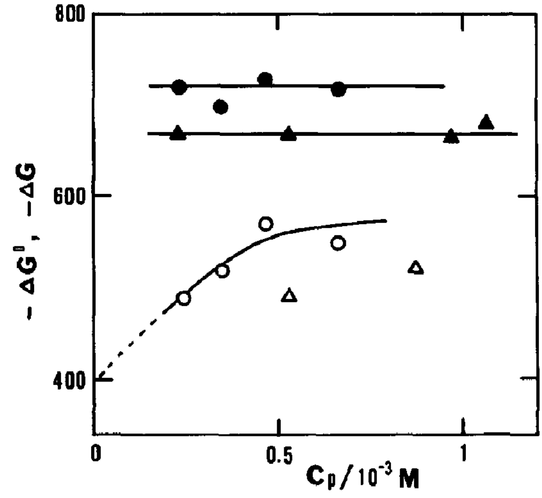

Fig. 8. Standard free energy change ΔG^0 and free energy of the whole solution ΔG (per residue) as functions of C_p in 50 mM NaCl solutions. Samples: $DP_w = 630$ (circles) and 360 (triangles). Open and filled symbols refer to the data at $t = 0$ and $t = \infty$ (1 day), respectively. ΔG^0 and ΔG are expressed in cal/mol.

that the stability of single folded-chain β-structure increases with chain length.

The value of ΔG from the titration curves corresponding to aggregation equilibrium is approx. -720 ± 20 cal/mol for the sample with $DP_w = 630$ and about -670 cal/mol for the other ($DP_w = 360$) for the C_p range flow 1×10^{-3} M. A value of -930 cal/mol was obtained in the previous study [11] for the same sample ($DP_w = 360$) at the high concentration of 9.6×10^{-3} M. At high concentrations, the contribution from aggregation is expected to increase. The difference between ΔG and ΔG^0 provided the contribution made by aggregation to the stability of the uncharged β-structure of poly(Cys(CH_2COOH)). This quantity was found to be $-(320 \pm 70)$ cal/mol for the sample with $DP_w = 630$.

4. Discussion

4.1. Diverse stabilities of folded-chain β-structures of various polypeptides

The stability of an uncharged β-structure relative to uncharged random coils is indicated by the standard free energy change ΔG^0. This quantity has been obtained as approx. -300 cal/mol for poly(L-tyrosine) [5,6] and has been reported to be approx. -120 cal/mol for poly(L-lysine) [7,8]. The value for poly(L-tyrosine) is reliable, while some reservation is necessary concerning the value for poly(L-lysine). The titration data for poly(L-lysine) were obtained under the condition that aggregation or even precipitation took place [7]. Nevertheless, this quantity has been often referred to as ΔG^0. It is interesting but puzzling at the same time that the value of ΔG^0 of poly(L-lysine) (aggregates) is even less negative than that for poly(L-tyrosine) (no aggregates). A possible interpretation of this problem was recently discussed [19]. The stabilities of the β-structure of L-valine (-900 cal/mol) and L-isoleucine residues (-515 cal/mol) were also evaluated from titrations on their copolypeptides with L-lysine [20,21].

Diverse values for the stability of the β-structure for polypeptides possessing different side chains are significant. Part of this variation arises from the different extents of uncertainty concerning different polypeptides in defining reference titration curves of the two conformations. Further work based on approaches other than potentiometric titration is required to clarify this point.

4.2. Stability of aggregates consisting of β-structures

In the present study, distinction between the contributions from unimolecular conversion and aggregation to the stability of uncharged aggregates of β-structure was made experimentally, although to an approximate degree. The contribution made by aggregation in the present case amounted to about $-(320 \pm 70)$ cal/mol for one sample. This result shows that the two contributions are comparable in the case of poly(Cys(CH_2COOH)). Furthermore, the value of this contribution is dependent on the concentration as well as on the molecular weight of the polypeptides. Possible formation of a hydrophobic region between stacked pleated sheets, as shown in the case of poly(L-lysine) [22,23], was rejected on carrying out an experiment based on using a fluorescent probe (unpublished data). According to the results, the fluorescence intensity of the probe increased, in accompaniment to formation of the β-structure of an alternating copolymer consisting of L-leucine and L-glutamic acid but did not change appreciably for the β-structure of poly(Cys(CH_2COOH)). Another possible reason for the stabilization is presented by the hydrogen bonding between carboxyl groups located in the inner region of stacked pleated sheets. This possibility was also discounted, since the presence of acetic acid or acetate ions hardly influenced the stability of poly(Cys(CH_2COOH)) [24]. At present, the contribution is considered to arise from aggregation of extended chains (formation of intermolecular β-structures).

It should be noted that stabilization due to aggregation also occurred in the case of poly(L-tyrosine), where the stability of aggregates (-500 cal/mol) [25] is much greater than that of molecularly dispersed β-structure (-300 cal/mol) [5,6].

4.3. Examination of the validity of the present procedure

For the present approach to be valid, conformational equilibrium of single nonaggregated chains should be established much more rapidly than the aggregation equilibrium. Our kinetic study in buffered solutions shows [10] that this is the case for pH ranges lower than about pH 4.65–4.70, which corresponds to a degree of ionization of random coils of 0.65–0.70. In unbuffered solutions encountered in the present study, the first event after the addition of acid is protonation without conformational change, resulting in random coils of the same degree of ionization as the whole solution. Consequently, the prerequisite for the present approach is satisfied for ranges of α below 0.65–0.70. At high charge densities, however, the data obtained are consistently interpreted if we assume that the reaction proceeds only through aggregation [10]. For a range of α between about 0.65–0.70 and 0.8 in the present study, this situation is likely to be the case. If the β-structures formed in this range of α mostly consist of extended chains, the present approach is still approximately valid over this range of α. In solutions containing various kinds of aggregates, however, molecularly dispersed folded-chain β-structures are also likely to exist. They would be produced, for example, through chain folding on the surface of aggregates followed by dissociation from it, as suggested in a study on poly(L-lysine) [26]. Only when these nonaggregated folded-chain β-structures are the predominant species does the present approach break down over this range of α. It should be noted, however, that the corresponding portion of the titration curve ($0.65–0.70 < \alpha < 0.80$) is not sufficiently long to affect the value obtained by more than 50 cal/mol. Taking this possibility into account, the final value of ΔG^0 was estimated to be $-(400 \pm 100)$ cal/mol.

In the present study, we have been concerned only with two extremes, $t = 0$ and $t = \infty$ and did not intend to draw information from the time course itself, since the kinetics in unbuffered solutions are rather complicated.

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