Potentiometric and Microcalorimetric Determination of the Thermodynamic Parameters of the Order–Disorder Transition of Poly(L-histidine) in Aqueous Solution[1a]

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ABSTRACT: Aqueous solutions of poly(L-histidine) (poly(His)) were studied by potentiometric titrations and microcalorimetric measurements in order to characterize the conformational changes the macromolecules exhibit when the hydrogen ion concentration of the solution is varied. The order–disorder conformational transition of poly(His), induced by lowering the pH, is characterized by a free energy change $\Delta G_c^\circ = 185$ cal/residue at 25°. The associated enthalpy change derived by potentiometric measurements is $\Delta H_c^\circ = 1.16$ kcal/residue and the average microcalorimetric value is $\Delta H_c = 1.0$ kcal/residue, at 25°. In agreement with the indications derived by the potentiometric data, 20% protonated poly(His) undergoes a thermally induced conformational transition at around 75° which was studied by means of circular dichroism (CD) measurements. A number of features of the potentiometric, microcalorimetric, and CD behavior of poly(His) in dilute aqueous solution relevant to its physicochemical and conformational characterization are discussed.

The conformational properties of poly(L-histidine) (poly(His)) in aqueous solution have been the object of a number of investigations.[2–4] It is presently well known that the polymer undergoes a conformational transition induced by changing the degree of protonation of the side chains. The CD properties of pure poly(His) and of random copolymers of L-lysine and L-histidine[4] unambiguously showed that the polymer assumes the random-coil conformation at high degrees of protonation of the imidazole side chains, while at low degrees of protonation an ordered structure is formed. On the basis of CD data one can safely exclude the right-handed α helix from the possible ordered forms. Myer and Barnard[3] interpreted the CD spectra of the poly(His) ordered conformation in terms of a β structure, this assignment, however, being complicated by the influence of the side-chain chromophores.

In the present paper we report the results of potentiometric titrations and of microcalorimetric measurements which allow a rather complete determination of the thermodynamic parameters relative to the transition ordered form → coil for uncharged poly(His) in dilute aqueous solution (0.02 *M* KCl and 25°).

Experimental Section

Materials. Pure poly(L-histidine) was prepared in our laboratory according to a procedure previously described.[2a] The sample used in this work was of the same stock of our previous paper,[4] and it contains 10% water of hydration.

Potassium chloride, potassium hydroxide, and hydrochloric acid were all reagent grade. Standard solutions of carbonate-free KOH have been prepared according to the literature.[5]

Equipment. Potentiometric titrations were performed with a Methrohm Model E388 potentiometer equipped with Beckman glass and calomel electrodes. A Methrohm precision micrometer buret, Model E457, was used.

Circular dichroism (CD) measurements were made with a Cary Model 60 spectropolarimeter equipped with a 6002 accessory unit and with a thermostatable cell assembly. Fused quartz cylindrical cells of 0.5- and 1-mm path lengths, with Suprasil windows, have been used.

Microcalorimetric measurements were performed at 25° with a LKB Model 10700 batch-type microcalorimeter.

Methods. (a) Potentiometry. Poly(His) solutions for potentiometric titrations were prepared by dissolving a weighed amount of polymer in water (or in KCl solutions of the required molarity containing the equivalent amount of HCl). The final polymer concentrations were in the range 0.0018–0.0035 *M* (residue). Higher concentrations do not allow titrations to be performed to low degrees of protonation of the side chains, since extensive polymer precipitation occurs. The titrations were carried out in a double-walled beaker, with circulation of thermostated water through the jacket, and 15 ml of solution was used in each experiment.

Electrode standardizations were performed with phosphate buffers and checked *before* and at the *end* of each titration. In general, the pH value of the buffer at the end of the titration was within 0.01 unit of the correct value. In a few cases the deviation was larger than this figure and the experiments were rejected. The reproducibility of different titrations curves on the same solution was always good and within 0.3%.

(b) Microcalorimetry. A weighed amount of poly(His) aqueous solution (c = 0.0037 *M* (residue) in 0.02 *M* KCl, degree of protonation 0.2) was introduced with a syringe in the larger side of the microcalorimeter cell. In the other side of the cell few microliters (5–10) of aqueous HCl (c = 0.042 *M* in 0.02 *M* KCl) were added prior to each measurements. With such additions $\Delta\alpha$ values of 0.030–0.050 were obtained. The rotation of the cells for mixing was carried out in such a way that after mixing the final solution would collect almost quantitatively in the larger side of the cell. In this manner, progressive additions of aqueous HCl could be made without taking out the solution and cleaning the cells each time. The accuracy in measuring the heat effects was better than 5%.

To avoid errors due to the slowness of the reaction in the α range connected with the transition, repeated mixings were made. In general, however, the first mixing was sufficient.

In these measurements the correction for dilution of the polymer was negligible while corrections for dilution of HCl were made. These corrections were of the order of 2×10^{-5} cal for a total heat ranging from 0.3×10^{-3} to 1.9×10^{-3} cal.

Treatment of Data. The fraction of deprotonated imidazole

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[1] (a) A preliminary account of this work was presented at the 5th Jerusalem Symposium, Jerusalem, April 4–10, 1972; (b) University of Padova; (c) University of Trieste.

[2] (a) K. S. Norland, G. D. Fasman, E. Katchalski, and E. R. Blout, *Biopolymers*, **1**, 277 (1963); (b) S. Beychok, M. N. Plumm, and J. E. Lehmann, *J. Amer. Chem. Soc.*, **87**, 3990 (1965).

[3] F. Meyer and D. Barnard, *Arch. Biochem. Biophys.*, **143**, 116 (1971).

[4] E. Peggion, A. Cosani, M. Terbojevich, and E. Scoffone, *Macromolecules*, **4**, 725 (1971).

[5] A. Albert and E. P. Serjeant, "Ionization Constants of Acid and Bases," Methuen Ltd., London, 1962, p 24.

groups in the potentiometric experiments is given by

$$\alpha = 1 - \frac{[(\mathrm{HCl}) - (\mathrm{KOH}) - (\mathrm{H}^+)_{\mathrm{free}}]}{[\mathrm{His}]_{\mathrm{total}}} \qquad (1)$$

where (HCl) is the molarity of the HCl added before the titration, (KOH) is the molarity of the added titrant, and $(\mathrm{H}^+)_{\mathrm{free}}$ refers to the molarity of free H^+.

The pH of a polyelectrolyte solution is given by[6-8]

$$\mathrm{pH} + \log \frac{1 - \alpha}{\alpha} \equiv \mathrm{p}K_{\mathrm{a}} = \mathrm{p}K_0 + \frac{0.434}{RT}\left(\frac{\partial G_{\mathrm{ion}}}{\partial \alpha}\right) \qquad (2)$$

$\partial G_{\mathrm{ion}}$ being the variation of electrostatic and conformational free energy consequent to an incremental change $\partial\alpha$ of the degree of ionization.

The area (determined by graphical integration) limited by the extrapolated and experimental curves of the random coil and of the ordered form in the $\mathrm{p}K_{\mathrm{app}}$ against α plots allows the determination of the standard free energy $\Delta G_0°$ per residue for the transition from *uncharged* ordered form to *uncharged* coil.

Results and Discussion

Potentiometric Titrations. The results of the potentiometric titration experiments on poly(His) in 0.02 *M* KCl carried out at different temperatures are shown in Figure 1. All curves, plotted according to the usual polyelectrolyte equation,[6] exhibit a marked inflection point, which reflects a conformational transition induced by changing the degree of protonation of the polymer side chains. It is important to point out that in the same range of α (degree of deprotonation) where the inflection point occurs, there is also a marked change in the CD properties of the solution.[4] Before evaluating the results in terms of thermodynamic parameters relative to the conformational transition of poly(His), some remarks have to be made. First of all, we have observed that, within the range of α values corresponding to the transition, the time required for the pH to reach the equilibrium value after each portional addition of titrant during the potentiometric titration was usually long. For instance, at 15° the equilibrium value of the pH was reached in 10 min or more, the higher the temperature the shorter being the time needed to reach the equilibrium. This behavior was recently observed in the titration of poly(L-lysine)[9] at high temperatures and of poly(L-tyrosine).[10,11] In one case,[9] this phenomenon was ascribed to the slow rate of formation of the β structure, while in the case of poly(L-tyrosine) the aggregation among β structures was considered responsible for the slow reaching of the equilibrium.[11] In the case of poly(His) we believe that this behavior, which occurs only in the transition region, is due to the slow rate of the conformational change and not to aggregation. The bases for this interpretation are in the fact that titration curves carried out in the concentration range 0.01–0.001 are overlapping, therefore excluding the presence of an important aggregation phenomenon, at least in the examined range of concentration.

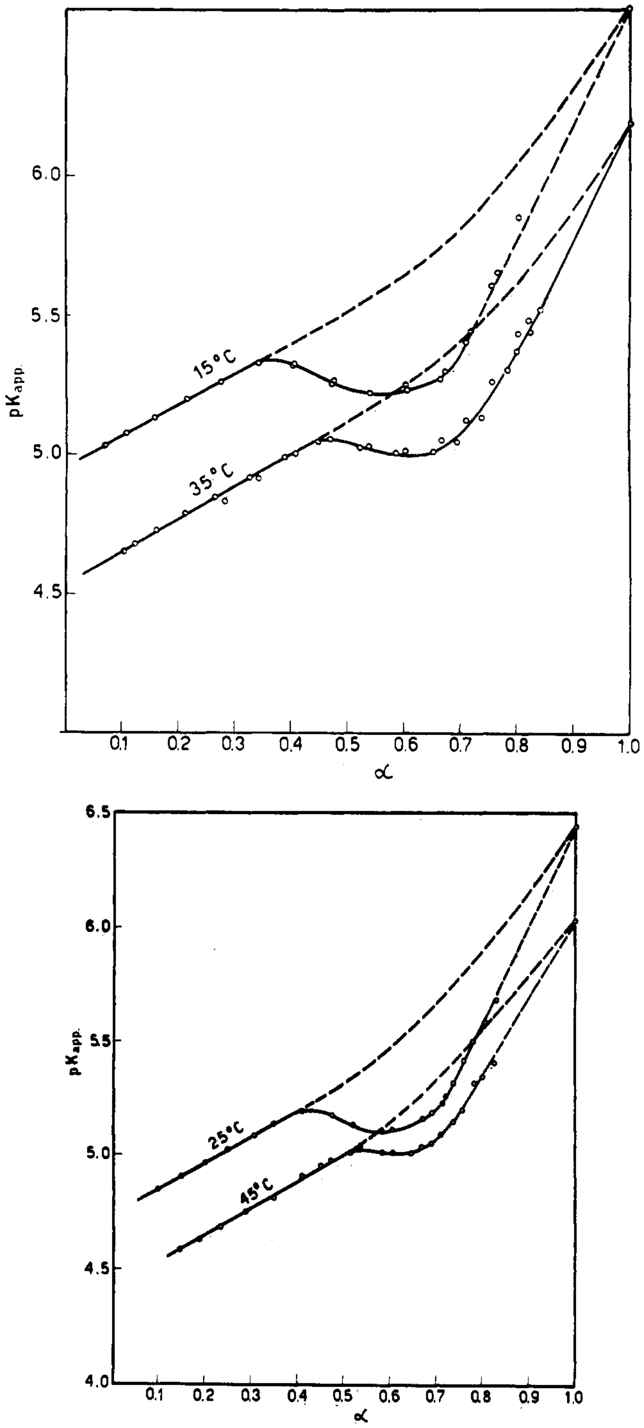

Figure 1. (Top) Potentiometric titration curves of poly(His) in 0.02 *M* KCl at 15 and at 35°. (Bottom) Potentiometric titration curves of poly(His) in 0.02 *M* KCl at 25 and at 45°.

A second main point of discussion concerns the difficulties in determining the $\mathrm{p}K_0$ values to which the titration curves have to be extrapolated. The difficulty concerns especially the extrapolation of the coil titration curves, since the extrapolation is very long and somewhat arbitrary. In principle, the $\mathrm{p}K_0$ of the coil and of the ordered form could even be not the same. In order to determine more accurately the $\mathrm{p}K_0$ values to which the extrapolations have to be made, we have carried out potentiometric titrations on the model compound Z-Leu-His-$\mathrm{OCH_3}$[12] in 0.02 *M* KCl and at the same temperatures at which the titration curves of poly(His) have been obtained. From the results of such measurements, which are presented in Table I, it was possible to determine precisely the

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(7) M. Nagasawa and A. Holtzer, *J. Amer. Chem. Soc.*, **86,** 538 (1964).

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(12) We are indebted to Professor L. Moroder of our institute for the gift of 200 mg of Z-Leu-His-O-$\mathrm{CH_3}$.

TABLE I
pK_0 OF THE MODEL COMPOUND Z-LEU-HIS-OCH_3 AT DIFFERENT TEMPERATURES IN 0.02 M KCl

Temp, °C	pK^0
15	6.59 ± 0.01
25	6.42 ± 0.01
35	6.20 ± 0.01
45	6.03 ± 0.01

TABLE II
$\Delta G_c°$ VALUES RELATIVE TO THE TRANSITION UNCHARGED ORDERED FORM → UNCHARGED COIL FROM POTENTIOMETRIC DATA AT DIFFERENT TEMPERATURES

Temp, °C	15	25	35	45
$\Delta G_c°$, cal/mol	212	185	140	100
$\Delta S_c° = 3.67$ eu			$\Delta H_c° = 1.16$ kcal/mol	

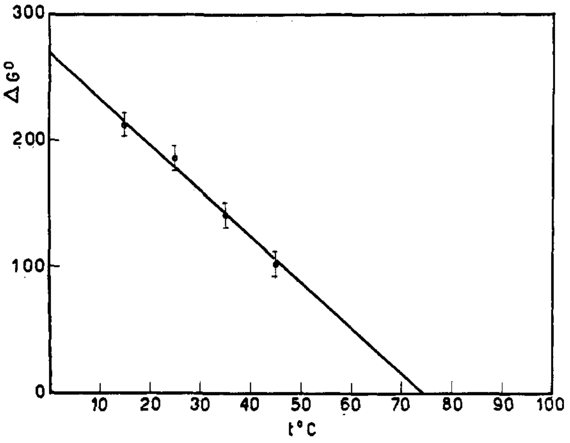

Figure 2. Free energy change (per residue) relative to the uncharged ordered form → uncharged coil transition as a function of temperature of poly(His) in 0.02 M KCl

pK_0 of an isolated imidazole group and its temperature dependence. On assuming that the pK_0 value of the imidazole group in the model compound is the same as in the coiled polymer at $\alpha = 1$, the final points of extrapolation of the coil titration curves of Figure 1 have been fixed.

From Figure 1 we also observe that the portions of the titration curves corresponding to the ordered form of poly(His) are almost linear and extrapolate with reasonable approximation to the same pK_0 of the coil titration curve.

In the evaluation of the $\Delta G_c°$ values relative to the transition uncharged ordered form → uncharged coil of poly(His) from the potentiometric titration curves, the main point of uncertainty is in the shape of the extrapolation curves.[6] Our data clearly indicate that the extrapolation of the coiled portion of the titration curves is not linear.

In all curves obtained at different temperatures, we draw curves having approximately the same shape, but in any case the limit of uncertainty is relevant. For these reasons the $\Delta G_c°$ values obtained from the areas bound by experimental and extrapolated curves must be considered approximate ones.

The $\Delta G_c°$ values relative to the conformational transition of poly(His) estimated from the potentiometric titration curves of Figure 1 are reported in Table II. From the temperature dependence of $\Delta G_c°$ (Table II and Figure 2), the associated $\Delta H_c°$ and $\Delta S_c°$ values have been calculated.

The results are $\Delta H_c° = 1.16$ kcal/mol and $\Delta S_c° = 3.65$ eu (at 25°). The relevant point which results from the plot of the

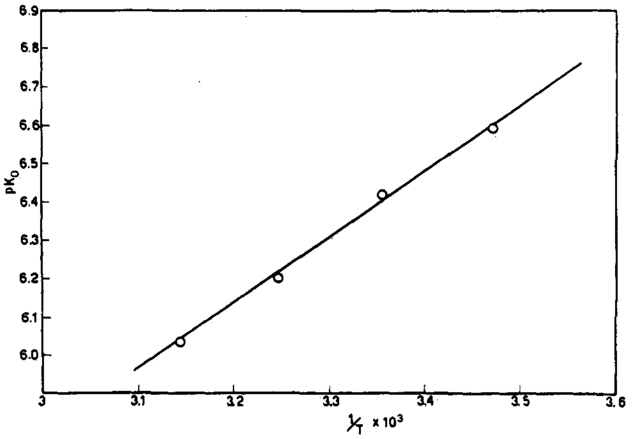

Figure 3. pK_0 in 0.02 M KCl of the model compound Z-Leu-His-OCH_3 as a function of the reciprocal of absolute temperature.

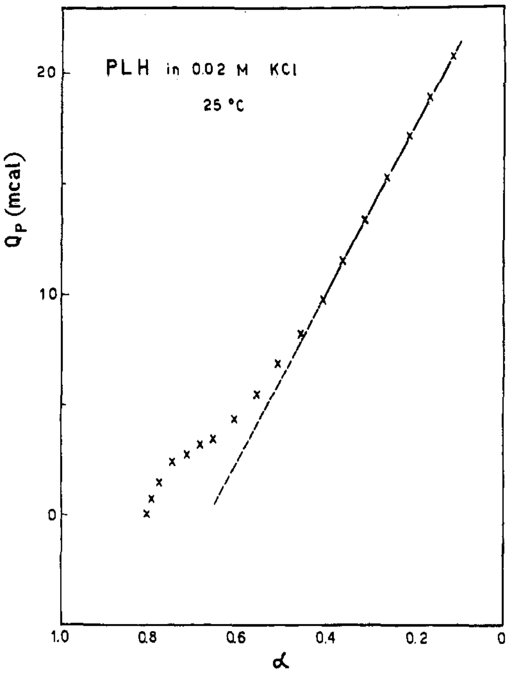

Figure 4. Integral heat of protonation Q_p starting with the polymer at $\alpha = 0.805$, as a function of α. 1.85532 g of poly(His) solution was used, corresponding to 6.901×10^{-6} mol of His.

Figure 2 is that $\Delta G_c°$ would become 0 at temperatures around 75° and would change sign above this temperature. In other words, below 75°, approximately, $\Delta G_c°$ is positive, indicating that the ordered form of uncharged poly(His) is *more* stable than the uncharged coiled form. At temperatures higher than 75° the reverse is true, the uncharged coiled form becoming the more stable conformation. On the basis of these results it is therefore predictable that uncharged poly(His) undergoes a *thermally* induced order–disorder transition. This prediction has been fully confirmed by CD measurements presented in a later section.

A further important point which is evident from the titration curves of the model compound and of poly(His) as well is that pK_0 depends on temperature (Table I). From the temperature dependence of pK_0 (Figure 3), the $\Delta H°$ relative to the dissociation process

$$HisH^+ \longrightarrow His + H^+$$

was determined. It was found that $\Delta H° = 7.85$ kcal/mol,

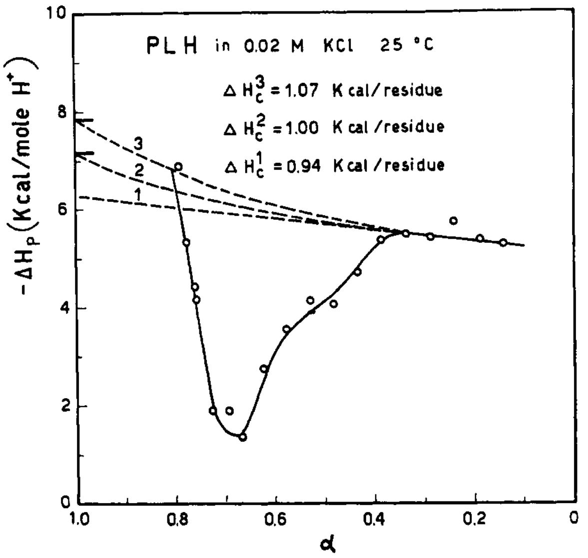

Figure 5. Differential heat of protonation as a function of α.

which is in fairly good agreement with literature thermodynamic data on the dissociation of imidazole compounds.[13] This value will be used in the determination of $\Delta H_c°$ relative to the conformational transition of poly(His) by calorimetric measurements described in the next section.

Microcalorimetric Measurements. The integral heat of protonation obtained with the method described in the Experimental Section starting with the polymer at $\alpha = 0.805$ is plotted in Figure 4 as a function of the (final) degree of *deprotonation*. Although this plot shows distinctly the range of α values where the transition of poly(His) takes place, it does not allow a precise evaluation of the heat of transition owing to the lack of experimental data before the occurrence of the conformational transition. In other words, while below α values of *ca.* 0.4 (state of coil conformation) the data fit a straight line, it is impossible to draw an analogous straight line for α values higher than 0.8 where the ordered conformation is stable. If one plots the differential heat of protonation as a function of the average degree of deprotonation, the curve of Figure 5 is obtained. In this case the heat of the conformational transition is measured by the area between the curve and a suitable base line. Again the impossibility of measuring the heat of protonation of the imidazole ring when the polymer is in its ordered state prevents drawing a good base line. In this case, however, it is possible to derive the most probable base line according to various methods, each of which will be discussed in what follows. All methods are based on the extrapolation of the microcalorimetric data obtained in the range of α values where the polymer is entirely random to $\alpha = 1$, where the polymer is completely ordered. The assumption which underlines these methods is that the heat of protonation of the histidyl group at $\alpha = 1$ is independent on the conformational state of the polymer. On the other hand, this assumption is analogous to that made in the case of the titration curves for the pK_0 value.

The first method is that of extrapolating directly with a straight line the data at low α values. In this case, a heat of protonation of the imidazole ring at $\alpha = 1$ (ΔH_p, $\alpha = 1$) of -6.30 kcal/mol and a ΔH_c of 0.94 kcal/residue is obtained. This extrapolation is represented by curve 1 in Figure 5. Base line 2 is obtained extrapolating to a value of ΔH_p, $\alpha = 1$, of -7.14 kcal/mol. This value was obtained by direct calorimetric measurements on L-histidine in 0.1 M KCl at 25° by Christensen, *et al.*[13] With this extrapolation a ΔH_c value of 1.00 kcal/residue is obtained.

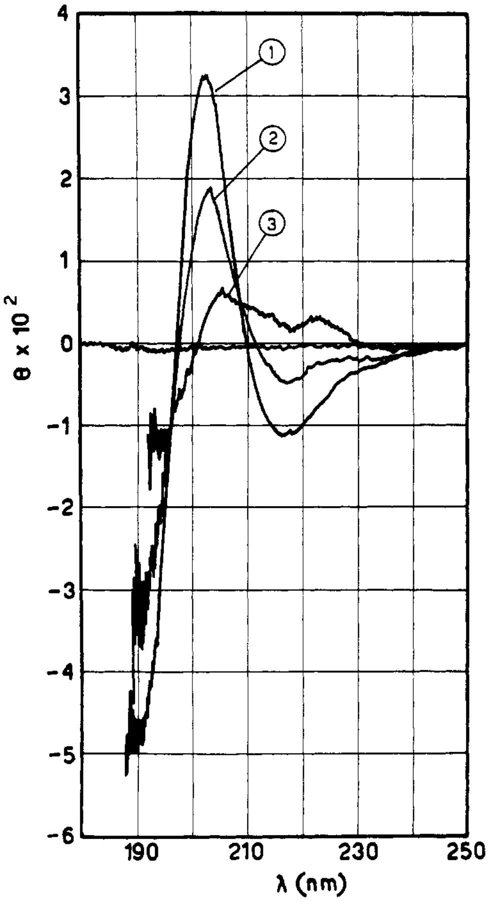

Figure 6. Some original CD spectra of poly(His) in 0.02 M KCl at different temperatures: (a) 25°, (2) 72.2°, (3) 96.5°. In the ordinate scale the measured ellipticity (degrees) is reported, from which the molar ellipticity [ϑ] and the molar circular dichroism $\Delta\epsilon$ can be calculated according to well-known equations. In this case the ordinate scale corresponds to the 0.1 sensitivity of the instrument. The polymer concentration was 1.84×10^{-3} M (residue) and the cell path length was 0.5 mm. The degree of protonation of the polymer side chains was 0.2.

Base line 3 is drawn extrapolating to a value of ΔH_p, $\alpha = 1$, of -7.85 kcal/mol obtained by potentiometric measurements at different temperatures on the model compound, as described in a previous section. This extrapolation leads to a ΔH_c of 1.07 kcal/residue.

The values of ΔH_c obtained by microcalorimetry have to be compared with the value of 1.16 kcal/residue obtained by the temperature dependence of potentiometric data. Taking into account the errors connected with the potentiometric method, the errors of the calorimetric one (essentially due to the uncertain choice of the base line) and the possible difference between ΔH_c and $\Delta H_c°$, the agreement between the two sets of data is rather good indeed. This fact is most gratifying in view of the eventually large unexplained difference in thermodynamic data, as derived by different authors by means of potentiometry and calorimetry, for conformational transitions of poly(amino acids).

For example, the enthalpy values for the $\beta \rightarrow$ coil transition of poly(L-lysine) reported by Pederson, *et al.*,[9] (potentiometry)

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(13) J. J. Christensen, R. M. Izatt, D. P. Wrathall, and L. D. Hansen, *J. Chem. Soc. A*, 1212 (1969).

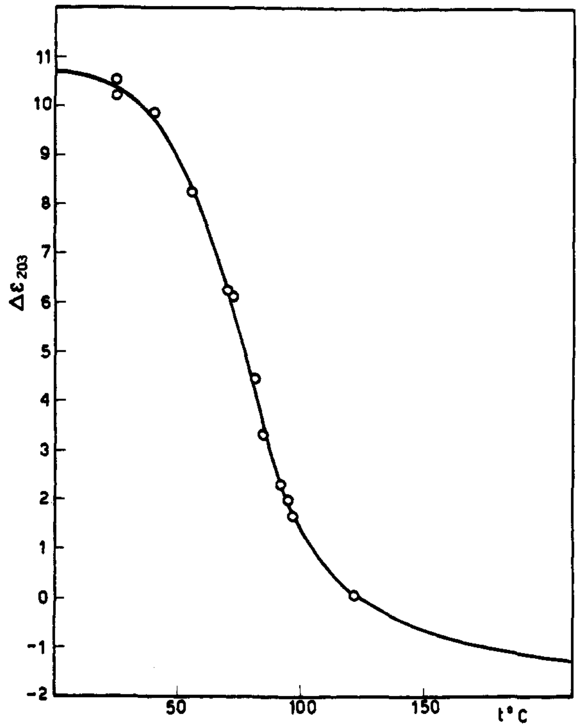

Figure 7. $\Delta\epsilon$ values at 203 nm as a function of temperature. The degree of protonation of the imidazole side chains in the polymer was 0.2.

and by Chou and Scheraga[14] (calorimetry) differ by nearly 2 kcal/mol.

The only other case of a poly(amino acid) $\beta \rightarrow$ coil transition which has been studied recently in detail is that of poly(L-tyrosine), for which Senior, *et al.*,[11] conclude that $\Delta G_c{}^\circ \approx 0$ at 25°!

Much more accurate calorimetric data appear necessary, in our opinion, to better elucidate the type of transitions among which we tentatively classify that exhibited by poly(His).

CD Measurements. In order to confirm the existence of a temperature-induced conformational transition of charge-free poly(His), predicted from the temperature dependence of $\Delta G_c{}^\circ$ (see Figure 2), CD measurements have been carried out in 0.02 *M* KCl and at different temperatures. In all cases the polymer concentration was the same as for potentiometric and CD measurements, and the fraction α of deprotonated imidazole groups was 0.80; measurements at higher α values were prevented by polymer precipitation. Some typical CD spectra recorded at different temperatures are shown in Figure 6. The plot of $\Delta\epsilon$ determined at 203 nm as a function of temperature (Figure 7) gives evidence of the predicted thermally induced conformational change. The transition appears to be not yet complete at 121.5°, which is the highest temperature at which the measurements could be performed. It is therefore not possible to calculate precisely the fraction of coiled poly(His) at each temperature and the half-transition temperature, since the CD pattern of uncharged poly(His) in the random-coil conformation is not known. In this connection we note that the CD pattern of coiled poly(His) in the protonated form has been reported in previous papers[3,4] which lend evidence for a change of the CD spectrum of *random poly(His) with the degree of protonation of the polymer side chains.* From Figure 7 it is only possible to locate roughly the half-transition point between 70 and 80°, which is in the range expected from the temperature dependence of $\Delta G_c{}^\circ$. However, even in the hypothesis that we could determine precisely the half-transition temperature, an absolutely good correspondence between predicted and experimental value cannot be expected for the following reasons. First of all, the uncertainty in the determination of $\Delta G_c{}^\circ$ values at different temperatures does not allow the prediction of an exact half-transition temperature. Secondly, the $\Delta G_c{}^\circ$ values calculated from potentiometric titration experiments refer to the transition *uncharged* ordered form $\rightarrow$ *uncharged* coiled form, whereas for solubility reasons the CD measurements at different temperatures have been carried out on 20% protonated poly(His).

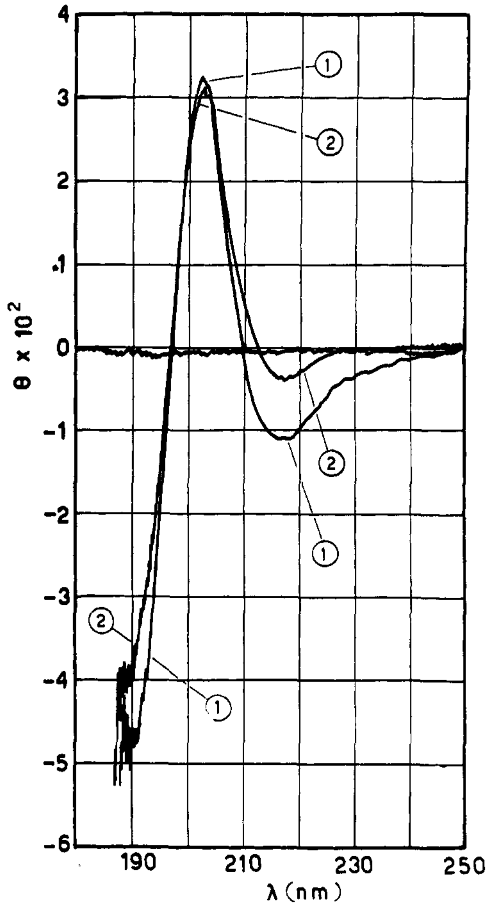

Figure 8. Changes in the CD pattern of poly(His) at $\alpha = 0.80$ after a complete thermal cycle. Curve 1 was recorded at 25°; curve 2 was recorded on the same solution cooled at 25° after heating at 120°. Curve 2 does not change with time after several hours.

During the CD measurements at different temperatures we became aware of a hysteresis phenomenon.

The poly(His) solution in 0.02 *M* KCl at $\alpha = 0.80$ heated at 121° and then cooled in about 0.5 hr at room temperature does not exhibit the initial CD pattern. This fact is more evident in Figure 8, which shows the CD spectra of the initial polymer solution at 25°, and of the same solution cooled at 25° after heating at 120°. It is evident that the band at *217 nm* does not reach the original value even several hours after the thermal cycle 25° $\rightarrow$ 121° $\rightarrow$ 25° has been completed. On the contrary, no hysteresis was found for the 203-nm band. Previous investigations have shown that the spectrum in the 220-nm region contains substantial contributions from the side-chain imidazole groups.[4] In the absence of added salts, this contribution appears to be slightly dependent on the conformation of poly(His).

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(14) P. Y. Chou, and H. A. Scheraga, *Biopolymers*, 10, 657 (1971).

Other authors[3] found a more marked dependence of the CD spectrum on the polymer conformation in the same spectral region. The discrepancy, besides the different poly(His) sample used by the two research groups, is partly imputable to the presence of salts. In fact, in 0.02 *M* KCl we also have found that the values around 220 nm depend on the polymer conformation.

From all this discussion, it appears that in the 215–225-nm spectral region there is a strong contribution to the optical activity from the imidazole side chains and that this contribution is somewhat conformation dependent. Turning back to the interpretation of the hysteresis of the 217-nm band, a possible interpretation is that, once the polypeptide molecules have been thermally denatured, a successive lowering of the temperature does allow the folding of the peptide backbone in the original ordered conformation, while the imidazole side chains are hardly able to find the final arrangement in the ordered structure of poly(His). This hypothesis could explain the normal behavior of the 203-nm CD band (which should mainly arise from peptide contributions) and the hysteresis of the 217-nm band (which should be mainly due to side-chain contribution). Such an interpretation could also account for the slow attainment of the equilibrium value of pH in the range of the conformational transition during the potentiometric titration experiments, caused by the slow arrangement of the imidazole side chains in the growing ordered structure of poly(His).

This feature appears well reconcilable with a random coil $\rightarrow \beta$ pH-induced conformational change of poly(His). However, pending direct unambiguous evidence in favor of such hypothesis it appears advisable to postpone any comparison between our set of results with those few reported in the literature for supposedly similar transitions.

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A New Theoretical Approach to the Problem of Solution Behavior of Branched Polymers

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ABSTRACT: In order to explain the solution behavior of star-shaped and comb-like polymers, we have extended Flory's and Orofino's calculations, taking into account the dependence of the interaction parameter χ on the local segment concentration. These calculations have been made assuming a gaussian distribution of segments around the center of mass. We have obtained the following results: (1) at Flory's Θ temperature, the second virial coefficient is different from zero and the coil is expanded; (2) the Θ_α temperature at which $\alpha = 1$ is different from the Θ_{A_2} temperature at which $A_2 = 0$, both of them being different from Flory's Θ temperature. These theoretical results account in the case of branched polymers for most of the yet unexplained results, especially the lowering of Θ_{A_2} and the discrepancies observed by several authors between the experimental dimensions and the corresponding calculated values; these discrepancies are due to an incorrect definition of the Θ dimensions. A comparison between experimental and theoretical values of Θ_α and Θ_{A_2} performed on 25 polymers prepared and studied in different laboratories is quite satisfactory.

Recent studies[1-7] carried out on the physical properties of model star and comb polystyrenes have shown that the behavior of such polymers in dilute solution cannot be described satisfactorily by the "two-parameter" theories—the two independent parameters being statistical segment *b* and excluded volume.

In these theories,[8-12] based on the single-contact approximation, the chain is assumed to obey random-flight statistics at the Θ temperature, a temperature at which simultaneously the second virial coefficient A_2 vanishes and the expansion factor α reaches unity.

The Θ temperature[13] or Flory temperature is thus a characteristic of a given polymer–solvent system, and is independent of the molecular weight of the polymer. However, in the case of the branched polymers we are studying in this paper, there are experimental results[1-7] which seem to be well established and which are in contradiction with the above-mentioned theory.

(1) The Θ temperature is always lower than that measured for linear homologs in the same solvent: it depends on the length and on the number of branches, *i.e.*, on the molecular structure of the polymer. When the degree of branching is low, there is coincidence in the values of Θ temperature for linear and branched polymers. There is only one reported exception to this rule: Berry[1] finds an increase of 2° for star polystyrenes in cyclohexane.

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