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THE CIRCULAR DICHROISM SPECTRUM AND STRUCTURE OF UNORDERED POLYPEPTIDES AND PROTEINS

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ABSTRACT

The evidence bearing on our revision of the early assignment of the circular dichroism spectrum of the maximally unordered polypeptide chain is reviewed. Our initial, as well as subsequent, studies are believed to support the proposed revision in assignment. This conclusion need not be modified in the light of recent work on water-soluble, non-ionizable polymers.

INTRODUCTION

Circular dichroism (CD) spectroscopy is well established as a useful method for identifying regular polypeptide chain conformations. This utility is based not only on experimental evidence [1-3], but also has a reasonable foundation in theoretical calculations of the CD spectra of the α-helix [4-7], β-structures [7,8-10], and to some extent, the polyproline helices [6,7,12].

The ultimate value of utilizing CD to study polypeptides and proteins, however, resides not only in the study of regular conformations, but in the identification of unordered structural components. These are relevant, for example, in specifying the complete structure of globular proteins [13], and they play a vital part in the conformational transitions polypeptides can undergo [14]. It is therefore of major importance that the CD identification of unordered polypeptide conformations be on as firm a footing as that of the ordered structures. Early studies did not lead to this kind of convincing assignment.

As a result of the cooperative nature of the pH-induced transitions in many properties of poly-L-glutamic acid (PGA) and poly-L-lysine (PL), it was assumed [15] that the CD spectra at the extremes of the transition were representative of those of the α-helix and of the "random coil". While there was ample evidence to support the assignment of the α-helix spectrum, there was no independent evidence that the CD spectrum of the charged form of these polypeptides was representative of a maximally unordered polypeptide chain (viz., a "random coil"). Yet this assignment formed the basis of the subsequent identification of unordered polypeptide chain structures, and it is still found in the current literature.

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The difficulty with the above CD assignment was first pointed out by us [16], and rests on two basic observations:

1) Charged polypeptides, in the absence of added salt, are relatively extended structures, as is well known from viscosity measurements [17], and therefore they cannot represent a set of chain conformations that are as unordered as might be achieved in the absence of charge repulsions. Preliminary calculations [18] suggested that such electrostatic interactions would favor a locally ordered structure with approximate three-fold left-handed helical symmetry, and this local conformation was designated an extended-helix (EH) structure [19]. It was shown not to be significantly modified by the presence of counterions [20].

2) Many polypeptide systems, under a variety of conditions better qualified for disordering the chain than charge repulsion, give CD spectra of a similar nature, yet different from that of charged PGA and PL. These include heat-denatured collagen [19,21] and solubilized feather keratin [19], aqueous $CaCl_2$ solutions of PL [19] and poly-L-proline (PP) [22], and several globular proteins under different conditions [19]. It thus seemed amply appropriate to revise the early assignment [15] of the CD spectrum of the maximally unordered polypeptide chain. Theoretical calculations of the CD spectrum of unordered polypeptide chains have not yielded identical results [23-26], yet in at least one calculation [26] there is consistency with such a revised assignment.

Although the above arguments seem reasonable to many investigators, there are claims in the current literature that the revised assignment is incorrect and that the original assignment should still stand. In this paper we review the additional evidence that has accumulated in favor of our revised assignment and discuss the merit of the arguments that dispute this revision. We also define as clearly as possible what is meant by an unordered polypeptide chain and indicate how its structure can be specified by CD spectroscopy.

UNORDERED POLYPEPTIDE CHAINS

It will be useful at this point to characterize what we mean by an unordered chain. The specification of an ordered chain, such as the α-helix or a β-structure, is relatively unambiguous: such a chain consists of a particular φ, ψ conformation at the C^α atom which is repeated over an extended portion of the chain. Unordered chains would then be those in which such a repeat in φ, ψ does not occur, that is, those " . . . spatial configurations which have no recurrent pattern of replication of bond conformations along the chain . . . " [27, p. 31].

Having made what seems to be a clear-cut distinction, we must, however, emphasize that we are dealing with matters of degree. The recurring φ, ψ values in an α-helix in solution cannot be *exactly* the same, but will be variable to the extent of the Boltzmann distribution about the minimum in the conformational energy map. This could imply variations of the order of 10° within a 1 kcal/mole energy range above the minimum [28-30]. Other structures which we think of as being ordered may have larger variations in internal angles. Thus, calculations of the conformational energy of PPII

[31-33] would indicate that a variation in ψ of the order of 60° is possible within 1 kcal/mole of the minimum (a somewhat smaller range is indicated if solvent interactions are taken into account [32]). The φ, ψ range which is potentially accessible to a polypeptide chain [28,29,34] is, however, significantly larger than indicated above. If experimental conditions permit the chain to sample substantially the entire accessible region, then the φ, ψ values will vary over widely different parts of the energy map (not being confined to a small region, as in the case of PPII), and we would expect the chain to approximate most optimally the condition of " . . . no recurrent pattern of replication of bond conformations along the chain . . . " [27, p. 31]. It seems to us that the term "unordered polypeptide chain" should be reserved for such a conformational state (assuming that it can be achieved or approached experimentally), and that it should be recognized that conditions which increasingly restrict the conformational space available to the chain are conditions which lead to greater degrees of order.

The existence of varying restrictions in φ, ψ space is very important, in that it can have different effects on the different experimental measures of chain conformation. The two main experimental techniques that have been used to infer the characteristics of chain conformation in unordered polypeptides have been hydrodynamic and spectroscopic. From the former an end-to-end length, or characteristic ratio, can be obtained, and this has been compared with values derived from theoretical calculations on perfect model systems [27]. From the latter, in particular CD spectroscopy, information is obtained about the local chain conformation. The differences between these two approaches cannot be overemphasized. The first gives a gross measure of chain conformation: a particular value of the characteristic ratio in no way defines uniquely the set of local conformations in the chain (the reverse is of course true). The second method is sensitive only to local conformation (and in an esentially unique fashion), being almost completely insensitive to long-range structure. It is therefore entirely possible for a polypeptide chain to exhibit particular local conformational restrictions, even though from a hydrodynamic point of view it may have characteristics of a "random coil". Therefore a certain amount of caution must be exercised in assuming the interdependence of arguments based on these disparate experimental techniques. We think, too, that CD provides a much more reliable indication of structure at the local level than do hydrodynamically derived parameters.

EVIDENCE FAVORING REVISED CD ASSIGNMENT

1. PGA

As we have noted [16], and as shown in Fig. 1, the CD spectrum of PGA well dialyzed so that no additional salt is present and at neutral or alkaline pH, consists of two bands: a weak positive band at 218 nm and a strong negative band at 198 nm. This spectrum is somewhat dependent on PGA concentration [16]: at lower concentrations the two bands are still observed, but the positive band is relatively more intense. The spectrum is significantly affected by all salts [16,19]. In 1.2 M NaCl or $LiClO_4$ a 218 nm band of about half the original intensity is obtained, accompanied

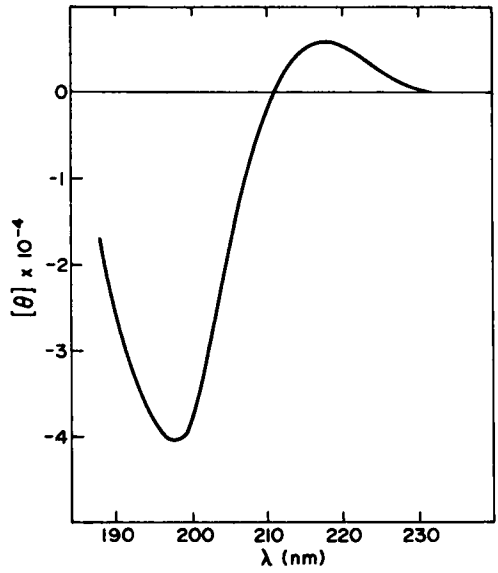

FIG. 1. Circular dichroism spectrum of poly-L-glutamic acid (Pilot, G97, MW~66,000), 0.06 to 2.0 g/liter, pH 8.0, 25°C

by the appearance of a weaker negative band near 235 nm (also observed by others [35]). When heated in 4.5 M $LiClO_4$ and cooled, the CD spectrum changes dramatically: the spectral structure in the 210-240 nm region disappears and the spectrum consists only of a broad and weaker negative band near 205 nm.

The above effects are consistent with a chain conformation in dilute salt-free solution which is maximally extended compared to conformations in concentrated solution and/or in the presence of salt. With respect to concentration effects, viscosities of salt-free polyelectrolytes increase with dilution, indicating more expanded chain domains [36]. (The extent of expansion in the salt-free state recently claimed for PGA [37] may have to be modified by considerations of viscosity measurements in very dilute solutions [38,39].) Thus, the intensified CD spectrum in the most dilute solution [16] should be representative of the local conformational state associated with maximal overall extension of the molecule. Similarly, the effect of salts in the shielding of charges indicates that an expanded conformation exists in the salt-free state (PGA is expected to behave as a "random coil" only at infinite ionic strength [40]). The significantly different CD spectrum observed in the presence, as opposed to the absence, of salts [16,19,35] (despite the inability of recent authors [41] to find such evidence in the literature!) indicates again that the salt-free spectrum cannot be taken as representative of the most completely collapsed state of the polypeptide chain, but rather that it should be assigned to a local conformational state associated with chain extension. As we have indicated [16,18-20], we think this (EH) state contains conformations close to that of a three-fold left-handed helix. The CD

spectrum produced by 4.5 M $LiClO_4$ goes beyond the effect of simple salts (4.5 M NaCl does not yield such a large change). We think this is a result of the particular nature of the interaction of $LiClO_4$ with the polypeptide backbone [42].

Another aspect of the CD spectrum of charged PGA has been examined recently and also shown to be consistent with the above assignment, viz., the effect of temperature [43]. When a salt-free solution of PGA is cooled to 5°C, both bands in the normal two-band spectrum intensify by about 25%. When the solution is heated above room temperature, both bands weaken and negative CD appears in the 230 nm region (at 55°C the ellipticity near 218 nm is essentially zero, a significant negative ellipticity being evident near 230 nm). The 40°C spectrum is very similar to that found in 1.2 M salt [19]. The effect of a 5° to 55° temperature range on the CD spectrum of PP is similar to the temperature effect on the spectrum of PGA between 5° and 25°C, viz., an affine change in band intensities. This, plus temperature effects on the spectra of ionized PL and other ionized polypeptides similar to those on PGA, suggests that the spectral changes have the same simple and obvious basis in all of these polypeptide systems. The populations of conformational states are clearly temperature dependent: at low temperatures most conformations will occur near the minima in the energy surface, and as the temperature is raised the probability increases that higher energy regions will be populated. The spectral change for PP is of necessity small since its conformational freedom is heavily limited [31-33]. However, the band intensification at lower temperature is completely consistent with experimental studies on rigid vs. flexible molecules [44] and with theoretical studies of the effect of helix length on the CD spectrum [4]. For PGA a much larger region is available in conformational space. Thus, although an analogous kind of confinement can occur at low temperatures as for PP, at high temperatures a much more extensive range of conformations is accessible and a concomitantly more significant change in the CD spectrum is possible. We see that, as expected, the effect of temperature and of salts is similar: both permit the chain to sample conformational space more broadly. To this extent they lead to more "random" chains than are present at low temperature in dilute, salt-free solutions.

It is appropriate to ask at this point whether the kinds of CD spectra referred to above for PGA in 1.2 M salt or at 55°C represent the most "random" or unordered conformations attainable. We think this is not so, having pointed out that PP in concentrated aqueous $CaCl_2$ solutions [22], PGA in 4.5 M $LiClO_4$ and PL in 4 M $CaCl_2$ [19], as well as fibrous and globular proteins under a variety of experimental conditions conducive to chain disordering [19], give a similar type of CD spectrum more appropriately assigned to an unordered chain. Such a spectrum is shown in Fig. 2 (for PP in $CaCl_2$), and is characterized by a single band at 200 ± 10 nm whose intensity depends on the particular system and conditions. (It should be noted that $CaCl_2$ is not influencing the chromophore, as is demonstrated by experiments on rigid model compounds [45].) An analogous spectrum has been predicted theoretically [26]. Its variability is an indication that " . . . the unordered chain represents no single structure but encompasses a range of structural possibilities" [19]. Different specific samplings of the overall conformational map will exist for different systems,

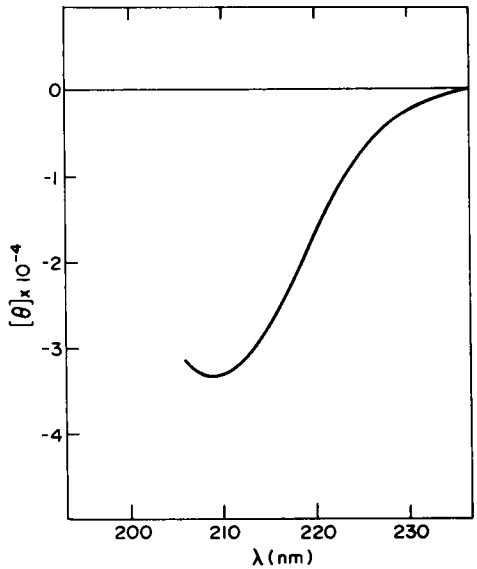

FIG. 2. Circular dichroism spectrum of poly-L-proline (Sigma, MW~7500), 0.1135 g/liter in 5.7 M $CaCl_2$, 25°C.

and these can show somewhat different CD spectra [26]. However, they have more in common with each other, in that they represent broad samplings of φ, ψ space, than they do with charged PGA, which represents a narrower distribution in conformational space [18,46]. This variability makes it highly unlikely that a single CD spectrum of a "random" polypeptide chain can be usefully assumed in analyzing for secondary structure in globular proteins, despite recent attempts to achieve this [47-49]. An important consequence of this is that, if we recognize the possible presence of unordered as well as EH states in polypeptide systems [43] (as well as α and β, of course), a variety of intermediate states is possible, with an associated span of CD spectra. It does not follow, for example, that a "225 nm trough" is characteristic of the unordered collagen chain [50,51]. We find that no such spectral characteristic persists in fully denatured collagen. The observation of such a feature [50,51] is more likely to indicate that some EH conformations exist in the presence of predominantly unordered structures, under the particular conditions of the experiment. It should be clear, incidentally, from experimental [19] as well as theoretical [26] studies, that the claim [50] that the residual absorption at about 200 nm in heat-denatured collagen can be attributed to " . . . some asymmetric structure . . . ", rather than arising from a substantially unordered chain, cannot be supported.

2. Poly (Glu-Ala)

Systems other than PGA have been studied, the results of which bear directly on the question of the CD assignment discussed above. Some of these involve side-chain

charge interactions, as in PGA, others do not. The latter reveal the important point [19,43,52] that a wide variety of factors can stabilize the EH state, and that it is therefore a structure of general importance. In this section we will discuss an example of the former type of system, poly (Glu-Ala).

Poly (Glu-Ala) exhibits a CD spectrum in its charged state (i.e., at alkaline pH) which is similar to that of PGA, except that upon extensive dialysis a minor negative band remains at 238 nm [53]. Upon addition of NaCl (at pH 10.5) the intensity of the positive band near 215 nm progressively decreases, the ellipticity at this wavelength being almost zero in 1.0 M NaCl [51]. In this respect the CD spectrum is much more sensitive to the concentration of NaCl than is the case for PGA [19], indicating that the conformation in the charged state is less stable than for PGA. At acid pH, the CD spectrum changes to that characteristic of substantial α-helical conformations [53]. The most interesting feature of these spectra is that when either the acid or the alkaline solution is heated to 90°C an identical CD spectrum is obtained, with a weaker negative band near 195 nm and no positive ellipticity at longer wavelengths. A small shoulder persists near 215 nm, but the spectrum is basically that which we assign to an unordered chain (with possibly some EH conformations remaining). The fact that elevated temperature causes both the alkaline and acid structures to change to the same conformational state constitutes strong evidence that the latter is a more unordered state, and therefore that the alkaline form must be a relatively ordered conformational state. The greater sensitivity of the EH form of poly (Glu-Ala) to heat and salts, as compared to PGA, seems to be explainable in terms of a greater conformational flexibility of the former charged polypeptide [54]. The necessity of adding methanol to develop the α-helix spectrum more fully [53] also indicates that the α-helical conformation of poly (Glu-Ala) is less stable than that of PGA.

The results of the study of poly (Glu-Ala) are thus not only consistent with our proposed CD assignment, but they provide important independent evidence in favor of such an assignment.

3. Poly (Ala-Gly-Gly)

The study of poly (Ala-Gly-Gly), PAGG, has provided another independent set of results which support our CD and structural assignments. Since side-chain charge interactions play no role in this polypeptide, this emphasizes the point that the EH conformation can also be stabilized by conditions other than charge repulsion [19, 43, 52].

The conformation of PAGG in the solid state depends on the solvent from which it is obtained: while some solvents produce a β structure, precipitation from dilute aqueous solution gives rise to a structure which is well characterized by X-ray diffraction as corresponding to that of polyglycine II, that is, a three-fold left-handed helix [55-57]. This form, PAGG II, is therefore topologically similar to the EH conformation. The CD spectrum of PAGG II can be obtained in aqueous solution and as a film cast from aqueous solution [58]. These spectra are essentially the same and are similar to that of charged PGA (Fig. 1). Since the cast film has been shown to have an EH conformation, it follows unambiguously that the EH conformation of a polypeptide chain gives a CD spectrum similar to that of Fig. 1.

The effects of heating [58,59] and salts [59] on the CD spectrum of PAGG II confirm our assignment of the spectrum of the unordered polypeptide chain. Heating an aqueous solution of PAGG II to 60°C results in a dramatic change in the CD spectrum: the positive peak near 213 nm disappears, and the negative band near 195 nm is considerably reduced in intensity. The spectrum is similar to that of an unordered chain (Fig. 2), and in fact strongly resembles that of heat-denatured collagen [19,21]. A similar spectrum is obtained in 6 M $CaCl_2$ solutions and from films cast from hot water [59]; infrared spectra of the latter are consistent with the polypeptide chain being predominantly in an unordered state. The experimental evidence on PAGG II thus provides unambiguous and independent support for our revised CD assignments.

Other similar sequential polypeptides exhibit CD spectra and temperature changes consistent with the above arguments concerning topology and the effect of temperature on it. These include poly (Pro-Ala-Gly) [60], poly (Ala-Ala-Gly) [61], and poly (Pro-Ser-Gly) [62]. The CD spectra of these various polypeptides are not identical to each other, but they are similar to that of charged PGA. This probably signifies that although the local topology is of an EH nature, variations exist in the detailed distributions in conformational space.

DISCUSSION

In the previous section we outlined the evidence that supports an assignment of the CD spectrum of charged PGA in solution to an EH type of conformation. Such a structure may also exist in the solid state [63]. Solid state and solution studies of PAGG II strongly support this assignment. These arguments also indicate that the CD spectrum of a maximally unordered polypeptide chain is one whose only significant structure is a relatively weak negative band near 200 nm.

Although these assignments have received general acceptance, there have been objections to them in the literature. These have been of two main kinds: one relates to whether two states (the α-helix and the charged PGA or PL) suffice to describe the CD spectra under all conditions, and therefore imply a special assignment of charged chains as true models of unordered polypeptides; and the other concerns the apparent contradictions implied by the CD spectra of the poly [N^5-(ω-hydroxyalkyl)-L-glutamine] polypeptides (the ethyl, PHEG, and propyl, PHPG, compounds being of most interest). We shall discuss each of these views in turn.

The CD spectra of PGA and PL, in the absence of salt, change as a function of pH. Myer [35] showed that intermediate spectra could be reproduced by superimposing appropriate fractions of the spectra observed at the two extremes of pH, and he concluded that two "conformationally distinct species" suffice to account for all intermediate states. The charged form was referred to as random, but no evidence was presented for this. That the pH-induced spectral changes can be explained by two conformational states (α-helical and EH in our interpretation) is neither surprising nor instructive as to the conformation of the charged form. This approach was pursued by Dearborn and Wetlaufer [65], who attempted to reproduce the spectra of PL in a variety of different salt solutions by using a two-state model similar to that

employed by Myer. It is difficult to evaluate the success of this exercise, since comparison curves were not presented but only "standard deviations", and these were for the not-too-sensitive interval of 212 to 228 nm. Nor is the meaning of their analysis clear, since they finally agree with us " . . . that 'random' proteins and 'random' homopolyamino acids show substantially different CD spectra . . . [for which they] do not at present have an explanation . . . ". In any case, they conclude that the charged form of PL is "unordered", basing this on the fact that the CD spectrum in this case is the same as that in 6 M GuHCl. They apparently assumed that the latter reagent leads to local chain disordering, an unsubstantiated conclusion. In fact we now believe just the opposite to be true [52]. Thus, neither of these studies provides any substantive objections to our proposed assignment.

A more perplexing problem is posed by the case of the water-soluble, nonionizable PHEG and PHPG polymers. The PHPG polypeptide was first synthesized by Arieh Berger and co-workers [65]. They presented its CD spectrum in methanol-water (where it is characteristic of that of an α-helix) and in water. In the latter solvent, it exhibits low negative ellipticity between 210 and 250 nm (the region of measurement), with a small inflection near 215 nm. They assumed this spectrum to represent primarily a random form of the polymer, a conclusion consistent with their hydrodynamic studies. It is interesting to note that Berger and co-workers understood the areas of relevance of these two techniques: "The information obtained from hydrodynamic studies relates to the molecular shape as a whole. For insight into the actual conformation of the molecule we rely mainly on optical rotatory measurements" [65, p. 647]. We find Berger's interpretation to be in substantial agreement with our revised assignments.

The basic difficulty arose with the study of PHEG [66]: the CD spectrum of aqueous PHEG was found to be qualitatively similar to PGA in its charged state, although the bands were weaker (about 1/3 as intense at 217 nm and about 2/3 as intense at 197 nm). Interestingly enough, PHEG showed a weak negative band near 235 nm which was about three times as strong as that seen in PGA (we now know that such a band is absent in salt-free PGA [16], or its presence may be due to residual blocking groups [37]). Since it was thought that charged PGA was "random", and it was assumed that PHEG in water was also in this state, it was supposed that both spectral results reinforced the assignment of the PGA spectrum to that of an unordered polypeptide chain [66,67]. This conclusion is unwarranted on several grounds; most important, we have essentially no independent evidence on the local chain conformation nor on the factors which determine it. The side chains of PHEG and PHPG contain peptide groups. We do not at present know how they interact with each other along the chain (via their dipoles), what the nature of their interaction is with the main-chain peptide groups (either via dipole-dipole interaction or hydrogen bonding), nor how solvent interaction may affect chain conformation. In fact, from the strong dependence of the CD spectrum on the side chain (i.e., PHEG vs. PHPG) it is clear that the main-chain conformation is very sensitive to side-chain structure. The point is that we have very little a priori basis for predicting the conformations of these water-soluble polymers, and, therefore, the presence of local order (for example of the EH

type) cannot be arbitrarily excluded. It is far more reliable to infer conformation from experimental behavior, and in this respect the early work on the effect of temperature and salts on PHPG [65] is consistent with our observations on other polypeptide systems and with our structural assignments. This is even more so in the case of recent studies on PHEG and PHPG [68]: the effects of temperature and salts are found to be cumulative, which in the light of our total evidence is consistent only with their exerting a disordering influence. (The apparently anomalous result obtained with PHPG in $CaCl_2$ is a consequence of the authors' neglect to go to high enough salt concentration [69]). Thus, rather than being " . . . difficult to rationalize the results reported here in terms of the work of Krimm and coworkers" [68, p. 733], we find the exact opposite to be the case. We also feel that the emphasis on characteristic ratio values [68,70] is misplaced. Not only are there important unresolved problems associated with the experimental and theoretical determinations of this quantity, but the characteristic ratio is in any case only a weak determinant of the details of the local conformational structure.

We therefore conclude that, while problems of detailed understanding remain in the analysis of the CD spectra of PHEG and PHPG, there is no fundamental difficulty in incorporating their behavior in our proposed assignment. In fact, it would be difficult to reach alternative conclusions. In summary, recent studies provide increasingly stronger support for our original assignments: the CD spectrum of charged PGA (Fig. 1) corresponds to a local conformation representative of a restricted region in φ,ψ space (designated an EH structure); the CD spectra of unordered polypeptide chains (Fig. 2) consist of a class characterized only by a relatively weak negative band in the 200 ± 10 nm region. The above conclusions are not modified substantially by the possibility that PP in $CaCl_2$ may contain some *cis*-imide bonds in addition to the predominantly *trans* linkages [71,72]. In the first place, the assignment of *cis* bonds in PP by NMR needs further substantiation: since the chemical shifts are as yet unexplained in detail, the possibility is not excluded that they do not arise directly from the *cis* bond but from some associated effect such as ring puckering [73], which may occur in the presence of salt even though the imide bond is *trans*. Secondly, the CD spectrum of PP in $CaCl_2$ cannot be explained on the basis of a combination of *cis* and *trans* structures: this would require a positive CD at 228 nm, which is not observed [22]. Finally, even if some *cis* bonds were present in PP, this is not pertinent to other polypeptides, where a *cis* configuration for the amide bond is energetically less likely. Since the CD spectrum of such polypeptides is similar to that of PP in $CaCl_2$, we consider the latter spectrum to be representative of those of unordered polypeptide chains.

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