Conformations of Poly(ethylene glycol) Bound Homooligo-L-alanines and -L-valines in Aqueous Solution

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Abstract: A conformational analysis of poly(ethylene glycol) bound *N-tert*-butyloxycarbonylhomooligo-L-alanines and L-valines to the octapeptides was performed in aqueous solution using circular dichroism. It was shown that the alanine and valine peptides may adopt β or statistical coil conformations depending upon chain length, concentration, temperature, ionic strength, presence of the N-blocking group, and pH. In addition, the β structures formed by the valine peptides are more stable than those formed by the corresponding alanine peptides. The origin of the positive dichroism near 215 nm in the homooligo-L-alanines and the effect of mono- and bifunctional poly(ethylene glycol) upon oligopeptide conformation are also discussed.

Introduction

Investigations of well-characterized, monodispersed, linear homooligopeptides have been extremely useful for the interpretation of conformational preferences of biologically active peptide molecules.[2-5] In particular, from the conformational analysis of a homologous series the minimum number of residues necessary for the formation of stable secondary structures can be determined. However, solubility problems have considerably limited the investigations in aqueous solution of homooligopeptides derived from α-amino acids with aliphatic hydrocarbon side chains.[6-10] Only the alanyl series could be examined above the dipeptide level (to the tetra- or hexapeptide depending upon the type of N- and C-blocking groups employed), but no ordered secondary structure was shown.[6-9]

To overcome this limitation, the liquid-phase method for peptide synthesis using poly(ethylene glycol) (PEG)[11] as the macromolecular C-terminal protecting group has been recently proposed by Mutter et al.;[12] in particular, the PEG-bound N-deblocked deca-L-alanine, examined by CD in water, exhibited a curve typical for a β conformation.

In this paper we describe the results of a CD study in aqueous solution, as a function of concentration and temperature, of the following three complete, monodispersed, PEG-bound homologopeptide series: *t*-Boc$\leftarrow$L-Ala$\rightarrow_{1-8}$Gly-OPEG, *t*-Boc$\leftarrow$L-Val$\rightarrow_{1-7}$Gly-OPEG, and *t*-Boc$\leftarrow$L-Val$\rightarrow_{2-8}$Gly-OPEG-M. Their N-deblocked derivatives have been also examined at different pH. In each series a single glycyl residue has been incorporated at the C-terminal end of the peptide chain as an internal standard in the amino acid analyses.[13] The alanyl series has been selected because of the well-established tendency of oligo-L-alanines to form right-handed α-helical and/or antiparallel β conformations;[2-7,12,14-19] the valyl series conversely, since oligo-L-valines are known to adopt a β conformation of very high stability, probably of the parallel-chain type.[2-4,14-17,19-21]

In addition, this study correlates with recent investigations devoted to clarify conformation and conformational stability of proteins when covalently linked to a water-soluble polymeric matrix.[22-24]

Experimental Section

Synthesis. The details of the synthesis of *t*-Boc$\leftarrow$L-Ala$\rightarrow_{1-8}$Gly-OPEG, *t*-Boc$\leftarrow$L-Val$\rightarrow_{1-7}$Gly-OPEG, and *t*-Boc$\leftarrow$L-Val$\rightarrow_{2-8}$Gly-OPEG-M are reported in ref 13 and 25. All compounds are chromatographically and analytically pure, with the single exception of *t*-Boc$\leftarrow$L-Val$\rightarrow_{7}$Gly-OPEG, which was shown to be contaminated by its lower homologue in the N-deblocked form ($\simeq$10%). PEG and PEG-M, molecular weight 10 000, were Hoechst (Frankfurt) products.

Circular Dichroism. CD spectra were recorded using a Cary 61 dichrograph and 0.5-mm, 1-mm, and 1-cm path length quartz cells. Dry, prepurified nitrogen was employed to keep the instrument oxygen-free during the experiments. Temperature was controlled by means of a hollow-walled, brass cell holder through which water was circulated. The temperature in the cell was determined using a Philips thermistor. No thermal deblocking of the N-terminal protecting group occurred at neutral pH, as shown by the absence of glycine in the amino acid chromatogram from a sample of *t*-Boc-Gly-OMe which was heated at 65 °C in water for 30 min and subsequently hydrolyzed with a 1 N NaOH solution. The absence of thermal degradation of the samples was confirmed by regeneration of the original CD spectra upon cooling.

The values are expressed in terms of $[\theta]_T$, total molar ellipticity. The Lorentz refractive index correction was not applied. The calibration was based upon $[\theta]_{290} = 7.840$ deg cm^2 dmol^{-1} for a purified sample of (+)-10-camphorsulfonic acid (Fluka) in 0.1% aqueous solution. The CD data represent average values from at least three separate recordings. The measurement at pH 9.0 was performed using a 5 mM Tris buffer.

Results

In Figures 1 and 2 the CD spectra of PEG- (or PEG-M-) bound homooligo-L-alanines and L-valines in aqueous solution are given; the influence of chain length, presence of the N-blocking group, and type of amino acid side chain, whether small linear (Ala) or β branched (Val), on the oligopeptide conformations may be deduced.

From a comparison of the CD spectra of Figure 3A with those of Figure 1B and the CD spectra of Figure 3B with those of Figure 2B the effect of a charged N-terminal residue may also be evaluated. From Figure 4 the type of β structure[26] which is formed in both series (interpeptide chain) is evident. The effect of heating and addition of neutral salts is apparent from Figures 5 and 6, respectively. Temperature, salt concentration, and pH are by another way a contribution to the study of the conformational stability of oligoalanines and valines.

Finally, Figure 7 presents the temperature-dependent salt-sensitive positive CD at 215 nm exhibited by H_2^+-$\leftarrow$L-Ala$\rightarrow_n$Gly-OPEG (n = 1-5) and supports the view that an order-disorder transition is not responsible for the changes observed in the CD spectra.[8,9,26]

All the experimental findings obtained (in part not shown in the figures) will allow us to discuss also the influence of mono- and bifunctional PEG upon oligopeptide conformation.[12]

Discussion

Since PEG and PEG-M contain only C-C, C-O, C-H, and

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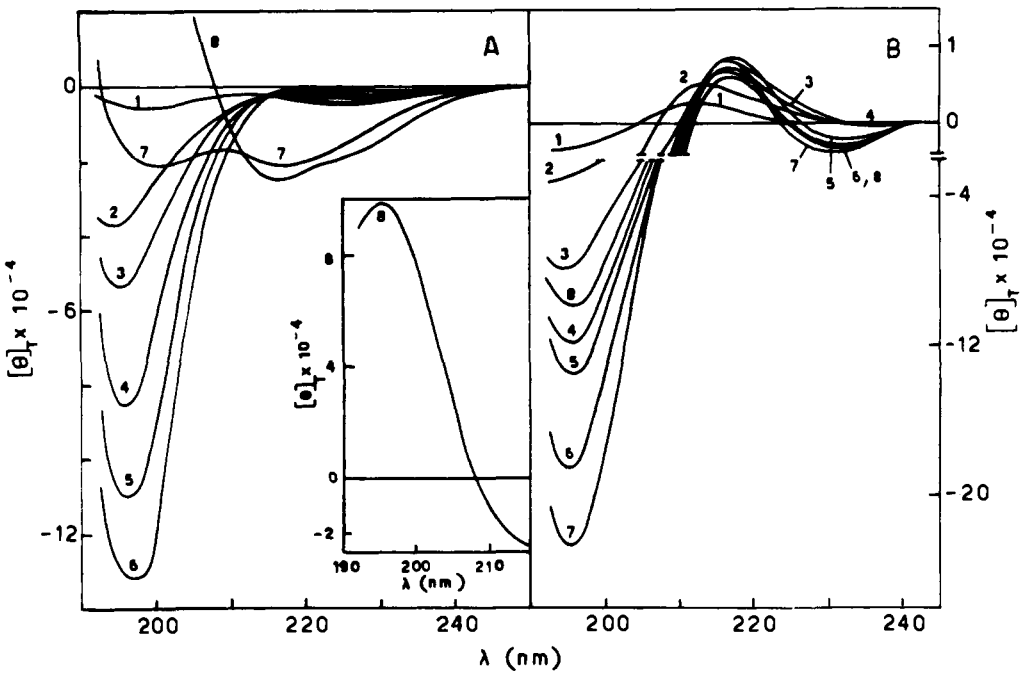

Figure 1. Circular dichroism spectra of *t*-Boc⟨L-Ala⟩$_n$Gly-OPEG in water (A) and H_2^+ ⟨L-Ala⟩$_n$Gly-OPEG at pH 3.5 (B); concn 3 × 10^{-4} M (n = 1–8).

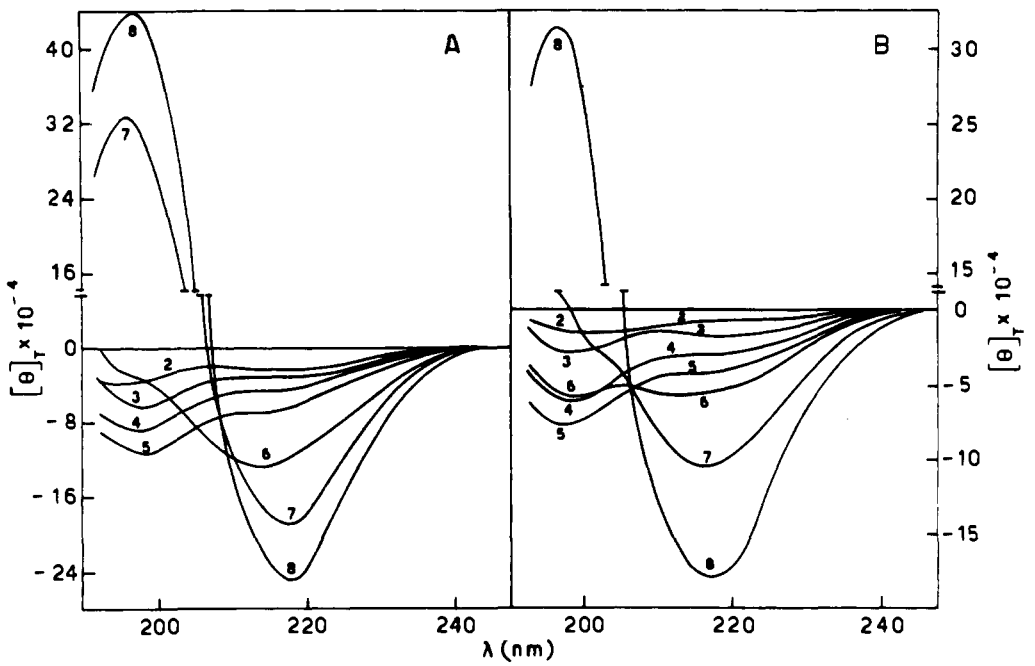

Figure 2. Circular dichroism spectra of *t*-Boc⟨L-Val⟩$_n$Gly-OPEG-M in water (A) and H_2^+ ⟨L-Val⟩$_n$Gly-OPEG-M at pH 3.5 (B); concn 10^{-4} M (n = 2–8).

O–H bonds, they have no electronic transitions above ≃180 nm.[12,26] Consequently, CD measurements in the region of peptide absorption (in particular, of n→π* and π→π* amide transitions at about 220 and 190 nm, respectively)[26] can be performed using polypeptide chains covalently linked to PEG or PEG-M. The compounds examined herein also have contributions to CD of the ester (C-terminal group) and urethane (N-terminal group) chromophores. However, by examining a complete series of homooligopeptides, it is possible to show changes in the spectral patterns as the chain length increases and directly correlate them with the onset of ordered secondary structures, the contribution to CD of N- and C-terminal chromophores being eliminated in using this approach.[3,8]

Influence of the Chain Length, Presence of the N-Blocking Group, and pH. The CD spectra in water (concn 3 × 10^{-4} M) of the lowest homologues of the N-protected alanine series (to Ala_6) are characterized by an intense negative maximum at 194–197 nm and a much weaker negative maximum at 225–230 nm, indicative of an essentially statistical coil conformation[26] (Figure 1A). The curve of Ala_6, conversely, showing a negative maximum at 216 nm, a crossover point at 208 nm, and a strong positive maximum at 196 nm, can be associated with a predominantly β conformation.[26] In the case of Ala_7 a complex CD pattern is visible, probably related to a mixture of unordered and β forms.[27] *t*-Boc⟨L-Ala⟩$_8$Gly-OPEG is the first fully protected monodispersed alanine oli-

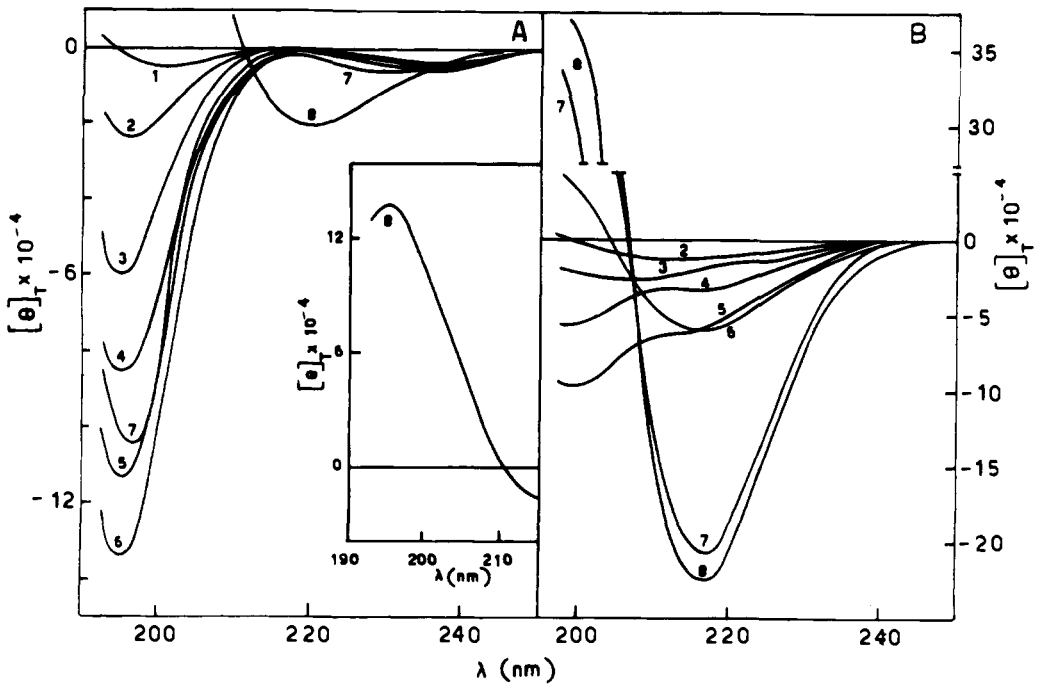

Figure 3. Circular dichroism spectra of H-(L-Ala)$_n$Gly-OPEG (n = 1–8) at pH 9.0, concn 3 × 10^{-4} M (A), and H-(L-Val)$_n$Gly-OPEG-M (n = 2–8) at pH 9.0; concn 10^{-4} M (B).

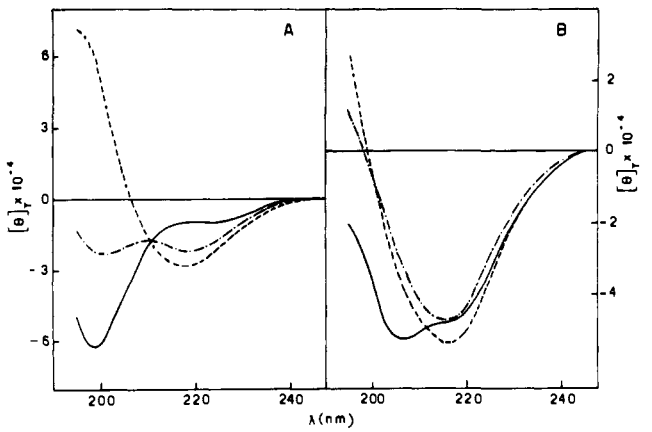

Figure 4. Circular dichroism spectra of t-Boc-(L-Ala)$_7$Gly-OPEG (A) and t-Boc-(L-Val)$_6$Gly-OPEG (B) in water at 10^{-4} (—), 3 × 10^{-4} (— ● —), and 5.5 × 10^{-4} M (- - -).

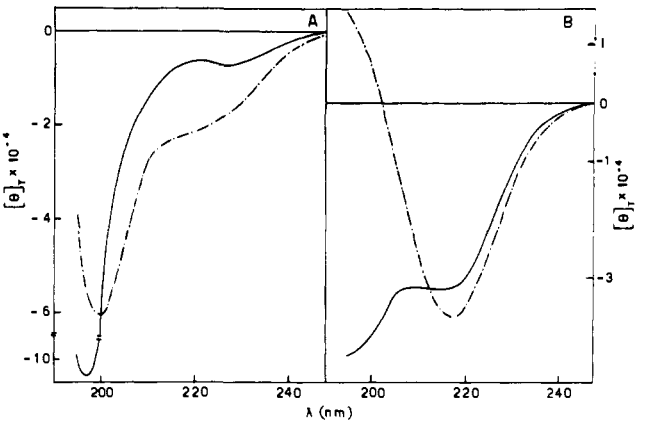

Figure 5. Circular dichroism spectra of t-Boc-(L-Ala)$_6$Gly-OPEG (A) and t-Boc-(L-Val)$_5$Gly-OPEG (B) in water (concn 5.5 × 10^{-4} M) at 20 (—) and 70 °C (— ● —).

gopeptide which exhibits an ordered secondary structure in considerable amount in water. This study confirms that the ordered secondary structure preferred by fully protected homooligoalanines in solvents of low capability to solvate its peptide chain is the β structure.[2-7]

From Figure 2A it is evident that also N-protected Val_7 and Val_8 (and in part Val_6) adopt the β conformation in water. The negative maximum, the crossover point, and the positive maximum appear at 217–218, 207–208, and 196–197 nm, respectively. The $[\theta]_{217}/[\theta]_{196}$ ratio is considerably different in Ala_8 and Val_8 (and Val_7), going from 0.25 in the former case to 0.56 in the latter. Thus, the well-known β-former character[2-4,14-17,19-21] of the valine residue stands out clearly also from the present study.

An inspection of Figures 1A and 2A in a comparison with Figures 3A and 3B, respectively, indicates that the presence of the N-protecting t-Boc group, although highly bulky and hydrophobic by nature, does not induce any remarkable change in the CD curves of oligoalanines and valines (with the single exception of Ala_7) in water.

A different situation appears in the N-deblocked oligoalanines and valines at acidic pH (Figures 1B and 2B) if compared to slightly alkaline pH (Figures 3A and 3B). The presence of the charged N-terminal residue reduces drastically the tendency of the oligopeptides to form the β structure (only Val_8 still assumes this ordered structure in a large amount when in the ammonium form). A second relevant feature at pH 3.5 which is absent at pH 9.0 is shown in the CD spectra of oligoalanines above 210 nm: in fact, a positive Cotton effect is visible at 215–217 nm, accompanied by a weaker negative Cotton effect near 235 nm. This observation was interpreted as resulting from a redistribution of conformation over the Ramachandran map due to changes in the interaction of peptide groups with ionic species.[8,9] This point is discussed in more detail below.

The CD curves of the N-protected and N-deblocked bi-

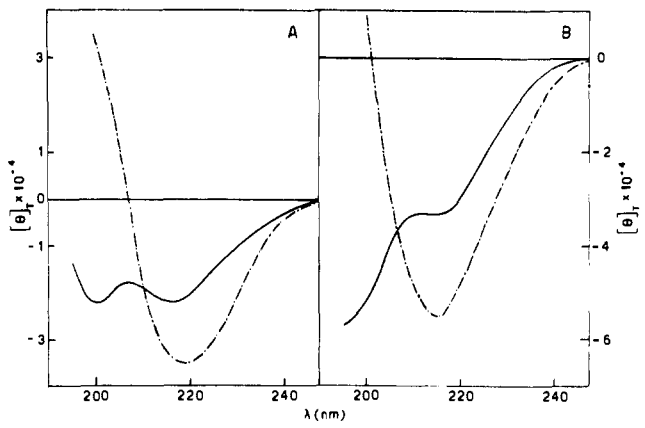

Figure 6. Circular dichroism spectra of *t*-Boc-L-Ala-$_7$Gly-OPEG (A) and *t*-Boc-L-Val-$_5$Gly-OPEG (B) in water (—) and in 2 M NaCl (— ● —); concn 3 × 10^{-4} M.

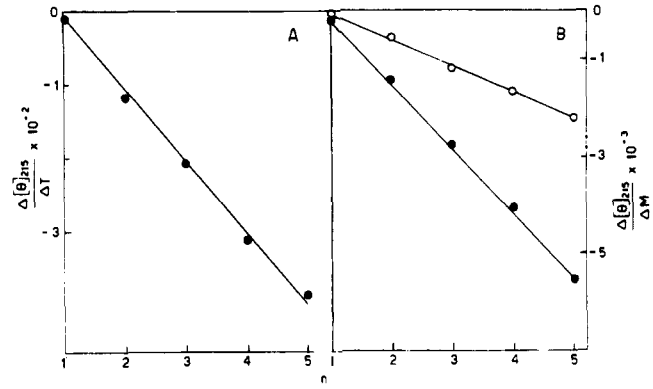

Figure 7. (A) Temperature effects in water, concn 5.5 × 10^{-4} M, at pH 3.5 on the positive circular dichroism band at 215 nm of H_2^+-L-Ala-$_n$Gly-OPEG (n = 1-5); (B) salt concentration effects in water, concn 3 × 10^{-4} M, at pH 3.5 on the positive circular dichroism band at 215 nm of H_2^+ L-Ala-$_n$Gly-OPEG (n = 1-5) (— o —, NaCl; — ● —, $NaClO_4$). The values in the ordinates were evaluated from spectra obtained (A) over the temperature range 20–70 °C and (B) over the salt concentration range 0–4 M.

functional PEG-bound valine peptides (not shown) are substantially similar to those reported in Figures 2 and 3B.

From the above data one may conclude that in water oligoalanine and valine chains tend to associate at the n = 6-7 stage. In this context, the occurrence of a N-terminal ammonium group, in contrast to the urethane *t*-Boc group, seems to decrease the extent of aggregated species.

Influence of Concentration, Temperature, and Ionic Strength. The conformational preferences of the three series from pentapeptides to the highest homologues in the N-protected and N-deblocked forms have been investigated at different concentrations, temperatures, and ionic strengths in water. Representative examples are illustrated in Figures 4-6.

In all the cases examined, if the oligopeptide concentration is increased, the conformational equilibrium will be driven more or less dramatically toward the β-associated form (Figure 4). This finding, although not excluding the occurrence of intrapeptide-chain hydrogen bonds as responsible for the observed ordered secondary structure, stressed the importance of interpeptide-chain hydrogen bonds in the stabilization of the β forms of these low molecular weight peptides.

The tendency to β-structure formation of oligoalanines and valines is enhanced by heating (Figure 5) and addition of sodium chloride (Figure 6). These results point to the important role of hydrophobic interactions on the stability of aggregated species. In fact, it is well established both theoretically and experimentally that increasing temperature and addition of neutral salts enhance the strength of hydrophobic interactions.[19,28-32]

Influence of Side Chain. From an inspection of the CD curves shown in Figures 1-3 it turns out clearly that under all the experimental conditions employed (presence or absence of the N-blocking group, and, in the latter case, acidic or alkaline pH) the critical chain length for the onset of a large amount of β structure in water is lower in the case of oligovalines than in oligoalanines. In addition, a careful comparison of the CD spectra of the highest homologues of the three series as a function of concentration, temperature, and ionic strength (in part not shown) clearly revealed that the stability of the β structures of oligovalines is higher than that exhibited by the alanine analogues. This is not surprising, in view of the well-known tendency of isopropyl groups (as in valine peptides) to form stronger hydrophobic interactions than methyl group (as in alanine peptides).[28,29,33]

The Positive CD Band near 215 nm of Alanine Homooligopeptides. In recent years the CD patterns of statistical coil peptide chains in water have been the center of much discussion.[26] In particular, the origin of the positive Cotton effect near 215 nm, and the assignment of the electronic transition responsible for it, is still a matter of controversy.[8,9,26,34] The presence of this band in water-soluble homooligo-L-alanines has been demonstrated by Goodman and co-workers[6] for MEEA-L-Ala-$_n$Mo (n = 3, 5, 6), by Mattice et al.[9,34] for Ac-L-Ala-$_n$OMe (n = 1-4), and by Toniolo and Bonora[7,8,35] for X-L-Ala-$_n$OMe (X = *t*-Boc, H, $^+H_2$; n = 2-4). Further evidence of the occurrence of this band in unordered linear homopeptides from α-amino acids with aliphatic hydrocarbon side chains is limited to *t*-Boc-L-Nva-$_2$OMe.[10] Mattice et al.[9,34] and Toniolo and Bonora[7,8] have also shown that heating and addition of neutral salts reduce the intensity of this positive CD maximum resulting eventually in a negative minimum.

Figures 1 and 3A indicate that in the PEG-bound homooligoalanines the positive dichroism is visible only in the N-deblocked series to Ala_8 at acidic pH. In Figure 7 we report the effect of temperature and neutral salt (NaCl and $NaClO_4$) concentration on the ellipticity at 215 nm of $^+H_2$-L-Ala-$_n$Gly-OPEG (n = 1-5). In all cases examined the $d[\theta]/dT$ and $d[\theta]/dM$ values are negative and fall on reasonably straight lines as a function of peptide chain length. In agreement with Mattice's results[34] the relative effectiveness of the two salts examined is $NaClO_4$ > NaCl. We have also confirmed[34] (results not shown) that the sensitivity of the CD band at 215 nm to temperature for a particular peptide is less in the salt solutions than in water; in addition, the temperature dependence is smaller in the presence of that salt ($NaClO_4$) that produces the larger changes in the ellipticity at 20 °C.

In summary, our CD results on short, flexible oligoalanines in the ammonium form demonstrate that it is not necessary to claim the occurrence of an ordered secondary structure for explaining the onset of a positive dichroism near 215 nm in water; rather it may be interpreted as resulting from a redistribution of a number of rotamers due to slight changes in the conformational energy of individual residues induced by temperature effects or coulombic interactions. We conclude that the statistical coil conformation of the small peptides, which is governed mainly by short-range interactions, undergoes conformational transitions in terms of flexibility and shape of the statistical coil by changing the temperature or solvent media; these transitions are reflected by the appearance/disappearance of a positive Cotton effect near 215 nm.

Influence of Mono- and Bifunctional PEG. The effect of PEG on the conformational preferences in water of hydrophilic homooligopeptides covalently bound to it has been recently examined by Mutter et al.[36,37] on *t*-Boc-L-Glu-$_n$Gly-OPEG (n = 7, 10, 14, 20). Identical CD spectra were obtained for

PEG-bound and C-deblocked peptides when measured under the same conditions. The addition of 10% PEG to the C-deblocked peptides also had no influence on the CD spectra. It was concluded that the polymeric C-protecting group does not interfere with the formation of an α-helical structure (starting at the heptamer stage) in water.

A direct comparison between the conformational properties of PEG-bound hydrophobic homooligopeptides and the same peptides not attached to the macromolecular support in water, i.e., the peptides with a preference for a β structure in that solvent, is difficult because of solubility problems and was not attempted so far. In this paper such a comparison is reported for homooligo-L-alanines, although limited to the lowest homologues of the series (Figures 1 and 3A and ref 7, 8, and 35). In all X$\leftarrow$L-Ala$\rightarrow_n$OMe (X = *t*-Boc, H, $^+H_2$; n = 2-4) peptides the positive dichroism near 215 nm is unambiguously observed.[7,8,35] However, in the analogous PEG-bound peptides this positive maximum is present only in the derivatives in the ammonium form (Figure 1B), being replaced by a negative minimum in the *t*-Boc (Figure 1A) and uncharged amino (Figure 3A) series. This finding indicates an influence, although certainly slight, of the polymeric C-protecting group on the conformational equilibrium mixtures of homooligo-L-alanines in water.

An interesting indirect comparison for the highest homologues of alanine and valine series is also possible. Previous studies from the Padova laboratory have shown that addition of water to a 2,2,2-trifluoroethanol solution of *t*-Boc$\leftarrow$L-X$\rightarrow_6$OMe (X = Ala, Val) results in the formation of interpeptide-chain β structures.[7,20] The tendency to assume an interpeptide-chain β structure in water for the *N*-*t*-Boc PEG-bound alanine and valine highest homologues is clearly apparent from the present investigation.

We conclude that in water the PEG group has only a minor influence upon the conformational equilibrium mixtures of the alanines lowest homologues, and, at least from a qualitative point of view, no influence upon the conformational preferences of the alanines and valines highest homologues. This is not unexpected, since, under the experimental conditions employed, PEG has a flexible, statistical coil structure, a specific interaction between peptide chains and PEG coil being very unlikely.[36-38] Finally, if the effects of mono- and bifunctional PEG are compared (in the valine series), no remarkable difference could be detected under the various experimental conditions tested. These conclusions strictly parallel those obtained by Lasch et al. on the conformational properties of chymotrypsin linked to a water-soluble, nonionic macromolecular support through a single covalent bond.[22] Thus, the CD investigations on PEG-bound polypeptide molecules in water are also relevant for the corresponding C-deblocked polypeptides.

Acknowledgment. One of the authors (M.M.) is grateful to the Deutsche Forschungsgemeinschaft for financial support. The expert technical assistance of Mrs. Maddalena Pernechele is also gratefully acknowledged.

References and Notes

(1) (a) Institute of Organic Chemistry, University of Padova; (b) Institut für Organische Chemie der Universität Tübingen.
(2) This work is part 55 of that series; for part 54 see G. M. Bonora, C. Toniolo, and M. Mutter, *Polymer*, in press.
(3) M. Goodman, C. Toniolo, and F. Naider in "Peptides, Polypeptides and Proteins", E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, Ed., Wiley, New York, N.Y., 1974, p 308.
(4) C. Toniolo and G. M. Bonora in "Peptides: Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1975, p 145.
(5) F. Naider and M. Goodman in "Bioorganic Chemistry", E. E. van Tamelen, Ed., Academic Press, New York, N.Y., 1977, p 177.
(6) M. Goodman, F. Naider, and R. Rupp, *Bioorg. Chem.*, **1**, 310 (1971).
(7) C. Toniolo and G. M. Bonora, *Makromol. Chem.*, **176**, 2547 (1975).
(8) C. Toniolo and G. M. Bonora, *Can. J. Chem.*, **54**, 70 (1976).
(9) W. L. Mattice and W. H. Harrison, III, *Biopolymers*, **14**, 2025 (1975).
(10) C. Toniolo, G. M. Bonora, and A. Fontana, *Bull. Soc. Chim. Belg.*, **84**, 305 (1975).
(11) The following abbreviations are used in the text: PEG, poly(ethylene glycol); PEG-M, poly(ethylene glycol monomethyl ether); *t*-Boc, *tert*-butyloxycarbonyl; Ac, acetyl; MEEA, 2-methoxy-[2-ethoxy(2-ethoxy)]acetyl; OMe, methoxy; Mo, morpholineamide; Ala, alanine; Val, valine; Gly, glycine; Nva, norvaline; Glu, glutamic acid; CD, circular dichroism.
(12) M. Mutter, H. Mutter, R. Uhmann, and E. Bayer, *Biopolymers*, **15**, 917 (1976).
(13) G. M. Bonora and C. Toniolo, *Makromol. Chem.*, submitted.
(14) A. Del Pra and C. Toniolo, *Macromolecules*, **11**, 793 (1978).
(15) C. Toniolo and M. Palumbo, *Biopolymers*, **16**, 219 (1977).
(16) J. S. Balcerski, E. S. Pysh, G. M. Bonora, and C. Toniolo, *J. Am. Chem. Soc.*, **98**, 3470 (1976).
(17) M. Palumbo, S. Da Rin, G. M. Bonora, and C. Toniolo, *Makromol. Chem.*, **177**, 1477 (1976).
(18) A. Fujie, T. Komoto, M. Oya, and T. Kawai, *Makromol. Chem.*, **169**, 301 (1973).
(19) J. C. Howard, F. Cardinaux, and H. A. Scheraga, *Biopolymers*, **16**, 2029 (1977).
(20) C. Toniolo, G. M. Bonora, and A. Fontana, *Int. J. Pept. Protein Res.*, **6**, 371 (1974).
(21) T. Komoto, K. Y. Kim, M. Oya, and T. Kawai, *Makromol. Chem.*, **175**, 283 (1974).
(22) J. Lasch, L. Bessmertnaya, L. V. Kozlov, and K. Antonov, *Eur. J. Biochem.*, **63**, 591 (1976).
(23) V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin, A. M. Klibanov, and K. Martinek, *Biochim. Biophys. Acta*, **522**, 277 (1978).
(24) A. Abuchowski, J. R. McCoy, N. C. Palczuk, T. van Es, and F. F. Davis, *J. Biol. Chem.*, **252**, 3582 (1977).
(25) H. Mutter, E. Bayer, and M. Mutter, *Makromol. Chem.*, submitted.
(26) R. W. Woody, *J. Polym. Sci., Macromol. Rev.*, **12**, 181 (1977).
(27) M. Rinaudo and A. Domard, *J. Am. Chem. Soc.*, **98**, 6360 (1976).
(28) H. A. Scheraga, G. Némethy, and I. Z. Steinberg, *J. Biol. Chem.*, **237**, 2506 (1962).
(29) G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
(30) M. Rinaudo and A. Domard, *Macromolecules*, **10**, 720 (1977).
(31) B. Davidson and G. D. Fasman, *Biochemistry*, **6**, 1616 (1967).
(32) A. M. Schwartz and G. D. Fasman, *Biopolymers*, **15**, 1377 (1976).
(33) R. E. Epand and H. A. Scheraga, *Biopolymers*, **6**, 1551 (1968).
(34) W. L. Mattice, *Biopolymers*, **13**, 169 (1974).
(35) C. Toniolo and G. M. Bonora, unpublished observations.
(36) M. Mutter, H. Mutter, and E. Bayer in "Peptides", M. Goodman and J. Meienhofer, Ed., Wiley, New York, N.Y., 1977, p 403.
(37) M. Mutter, *Macromolecules*, **10**, 1413 (1977).
(38) K. G. Ingham, *Arch. Biochem. Biophys.*, **184**, 59 (1977).