Macromolecules
Volume 19, Number 4 April 1986
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Macromolecular Analogues of the Copper(II) Binding Site of Human Serum Albumin. 3. Synthesis, Conformation, and Ion Binding Properties of Glycylglycyl-α,γ-diaminobutyric Acid Derivatives of Poly(L-lysine)

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ABSTRACT: In the attempt to prepare macromolecular analogues of the Cu(II) binding site of human serum albumin, poly(L-lysine) has been derivatized by covalent binding of the tripeptide sequence Gly-Gly-Dab (Dab = α,γ-diaminobutyric acid) to side-chain ϵ-amino groups. Polymeric adducts have been prepared with 50% and 100% side-chain modification. The conformational and Cu(II) or Ni(II) binding properties of the derivatized polymers have been investigated by absorption and circular dichroism (CD) techniques. It was found that in aqueous solution at pH $\geq$ 12 the 50% modified polymer folds into the right-handed α-helical conformation, while the 100% modified polymer remains in a random structure. Both polymers interact strongly with Cu(II) and Ni(II) ions. In aqueous solution at neutral pH complexes are formed in which each tripeptide chelating unit binds one metal ion. The spectroscopic properties in the visible–near UV absorption region indicate that the structure of the metal complexes is different from the one previously observed with Gly-Gly-His modified poly(L-lysine).[1] Our results are compatible with a structure in which the Gly terminal amino group and the three consecutive deprotonated peptide nitrogens of the side chain are coordinated to the metal ion. Complex formation causes folding of the main chain into the right-handed, α-helical conformation even in the case of the 100% modified polymer. These findings suggest the presence of stabilizing interactions among the side chains of the complexes.

Introduction

In a previous paper we described the synthesis, conformation, and binding properties toward Cu(II) and Ni(II) ions of poly(L-lysine) derivatives containing the tripeptide sequence Gly-Gly-His covalently bound to side-chain ϵ-amino groups.[1,2] Such derivatized polymers can be considered macromolecular analogues of the Cu(II) binding site of human serum albumin (HSA).[3-5] Indeed, it has been shown that the isolated tripeptide *N*-methylamide and its macromolecular adducts, in aqueous solution at neutral pH, exhibit strong affinity toward Cu(II) and Ni(II) ions, with binding constants of the same order of magnitude as those of the native protein.[2,3] In general, it is known that the presence of a His residue at the third position in tri- and tetrapeptide sequences enhances the affinity toward Cu(II) and Ni(II) ions.[6] This effect is due to the presence of the imidazole nitrogen, which favors coordination via a three-chelate ring system.[6] In the case of the sequence Gly-Gly-His, the N-terminal amino group, two consecutive, deprotonated peptide nitrogens, and one imidazole nitrogen are present at the coordination sites of the metal ion, in a square-planar geometry (Figure 1). In a previous investigation on the ion-binding properties of homopolypeptides,[7-9] we observed that the residue of α,γ-diaminobutyric acid (DAB) can be equivalent to a His residue at the effect of Cu(II) coordination. Also in this case in fact, a stable, hexatomic chelate ring structure is possible, in which the γ-side-chain amino group of Dab replaces the imidazole nitrogen of His in the coordination sphere of the metal ion. These findings suggest the possibility that the sequence Gly-Gly-Dab has binding characteristics very similar to those of the sequence Gly-Gly-His and could therefore represent a new analogue of the Cu(II) binding site of HSA. To test this possibility we have derivatized lysine polymers by covalent binding of the sequence Gly-Gly-Dab to ϵ-side-chain amino groups. In this paper we report the synthesis of polymers with side chains modified to a different extent and the results of conformational and binding studies toward Cu(II) and Ni(II) ions.

Abbreviations

The amino acids used were of L configuration. Standard abbreviations for amino acid derivatives are according to the suggestions of the IUPAC–IUB Commission on Biochemical Nomenclature *Biochemistry* **1972**, *11*, 1726–1732.

Other abbreviations used are Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Adoc, adamantyloxycarbonyl; OSu, *N*-hydroxysuccinimide ester; HONHS, *N*-hydroxysuccin-

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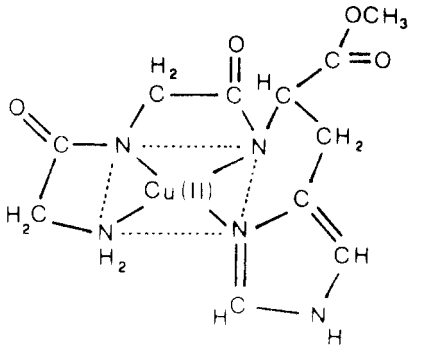

Figure 1. Structure of the H-Gly-Gly-His-O-CH_3–Cu(II) complex.

imide; DCCI, dicyclohexylcarbodiimide; NCA, *N*-carboxyanhydride; THF, tetrahydrofuran; TFE, 2,2,2-trifluoroethanol; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetracetic acid; CD, circular dichroism, TLC, thin-layer chromatography.

Experimental Section

Materials. $CuCl_2$ (purum, Merck) and $Ni(ClO_4)_2 \cdot 6H_2O$ (purum, p.a., Fluka) were used without further purification. KH_2PO_4 and $K_2HPO_4 \cdot 3H_2O$ were Carlo Erba RP products. The amino acid derivatives Z-Gly-OH, Z-Gly-OSu, Boc-Gly-OH, and Boc-Gly-OSu were prepared according to the general procedures described in the literature.[10-12] Poly(L-lysine) hydrochloride was prepared by the polymerization of N^{ϵ}-Z-Lys-NCA, followed by deblocking of the side-chain protecting groups via anhydrous HBr, according to the literature.[13]

[Dab(Adoc)]$_2$Cu. To a solution of 2HCl·H-Dab-OH (30 g, 157 mmol) in 320 mL of 1 M NaOH (320 mmol) was added 21.7 g (87.0 mmol) of $CuSO_4 \cdot 5H_2O$. The solution was left stirring, at the boiling point, for 15–20 min. After cooling at room temperature, 26.3 g (0.314 mol) of $NaHCO_3$ was added under stirring. The solution was cooled to 0 °C and treated with 37.4 g (0.189 mol) of 1-adamantyl fluoroformiate (Adoc-F) in dioxane (120 mL). The mixture was left stirring at room temperature overnight. The solid residue was recovered by filtration, repeatedly washed with cold water and diethyl ether, and finally dried in vacuo: yield 43.3 g (84.3%); $R_F^{I} = 0.43$.

H-Dab(Adoc)-OH. [Dab(Adoc)]$_2$ Cu (43.3 g, 66.2 mmol) was treated, under vigorous stirring, with EDTA disodium salt (37 g, 99.5 mmol) in 400 mL of water. To this mixture was added 300 mL of 1-butanol, and the pH was adjusted to 4 with 1 N HCl. The aqueous phase was washed with 1-butanol and the organic layer with EDTA to decoloration. The solid product, recovered after concentration of the organic solution, was repeatedly washed with diethyl ether and finally dried in vacuo: yield, 34.8 g (88.7%); $R_F^{I} = 0.52$; mp 216–218 °C; $[\alpha]^{20}_D = -24°$ (c = 1, methanol). Anal. Calcd for $C_{15}H_{24}N_2O_4$ (296.369): C, 60.80; N, 9.45; H, 8.16. Found: C, 59.87; N, 8.74; H, 8.38.

Z-Gly-Dab(Adoc)-OH. H-Dab(Adoc)-OH (20 g, 67.5 mmol) was dissolved in 600 mL of an aqueous solution containing 67.5 mL (67.5 mmol) of 1 N NaOH and 4.81 g (57.3 mmol) of $NaHCO_3$. The pH of the mixture was ~9. To this solution, cooled to 0 °C, was added 17.5 g (57.3 mmol) of Z-Gly-OSu dissolved in 600 mL of dioxane. The mixture was left stirring, at pH 7.5–8, at 0 °C for 5 h and finally at room temperature overnight. After evaporation of the dioxane, the aqueous solution was extracted twice with ethyl acetate, acidified at pH ~3 with 2% $KHSO_4$, and again extracted with ethyl acetate. The organic layer was first washed with a saturated solution of NaCl, washed with water until neutrality, dried over Na_2SO_4, and finally evaporated. The solid residue was recrystallized from diethyl ether/petroleum ether: yield, 22.8 g (81.7%); $R_F^{I} = 0.79$; $R_F^{III} = 0.12$; mp 75–78 °C; $[\alpha]^{20}_D = -45°$ (c = 1, methanol). Anal. Calcd for $C_{25}H_{33}N_3O_7$ (487.558): C, 61.58; N, 8.62; H, 6.82. Found: C, 61.53; N, 8.41; H, 7.02.

Amino acid ratio of the acid hydrolysate: Gly 0.99 (1); Dab 1.01 (1).

Boc-Gly-Gly-Dab(Adoc)-OH·H_2O. Z-Gly-Dab(Adoc)-OH (10.2 g, 21 mmol) was hydrogenated in a mixture of methanol (300 mL)/acetic acid (60 mL) in the presence of 10% palladized charcoal at room temperature and atmospheric pressure. The progress of the reaction was followed by TLC ($R_F^{I} = 0.27$ for the free peptide). At the end of the reaction, water was added to dissolve the free peptide. The catalysts were removed by filtration on Celite, and the filtrate was evaporated to dryness. The solid residue was triturated with diethyl ether, filtered, washed with the same solvent, and dried in vacuo: yield, 7 g (79.7%).

CH_3COOH·H-Gly-Dab(Adoc)-OH (7 g, 17 mmol) was dissolved in 250 mL of an aqueous solution containing 34 mL (34 mmol) of 1 N NaOH and 1.2 g (14 mmol) of $NaHCO_3$. The pH was ~9. To this solution, cooled to 0 °C, was added 3.9 g (14 mmol) of Boc-Gly-OSu dissolved in 250 mL of dioxane. The mixture was left at 0 °C for 5 h under stirring, and then at room temperature overnight. After evaporation of the dioxane, and adjustment of the pH of the aqueous solution to ~9, the aqueous solution was extracted twice with ethyl acetate, acidified at pH ≃3 with 2% $KHSO_4$, and again extracted with ethyl acetate. The organic layer was first washed with a saturated solution of NaCl, washed with water until neutrality, dried over Na_2SO_4, and finally evaporated. The solid residue was triturated with diethyl ether, filtered, and dried in vacuo. The product crystallizes with one molecule of water: yield, 6.3 g (85.1%); $R_F^{I} = 0.77$; $R_F^{II} = 0.81$; mp 115–118 °C; $[\alpha]^{20}_D = -4.6°$ (c = 1, methanol). Anal. Calcd for $C_{24}H_{40}N_4O_9$ (528.609): C, 54.53; N, 10.59; H, 7.62. Found: C, 54.41; N, 10.55; H, 7.52.

Amino acid ratio of the acid hydrolysate: Gly 1.94 (2); Dab 1.05 (1).

2CF_3COOH·H-Gly-Gly-Dab-OH. Boc-Gly-Gly-Dab(Adoc)-OH (0.135 g, 0.26 mmol) was dissolved in 10 mL of 95% trifluoroacetic acid (containing 5% of water) and left stirring for 2 h. The solvent was then evaporated and the residue dissolved in water. The insoluble material was filtered and the aqueous solution lyophilized: yield, 0.119 g (quantitative yield); $R_F^{II} = 0.2$; mp 85–88 °C; $[\alpha]^{20}_D = -34°$ (c = 1, water).

Amino acid ratio of the acid hydrolysate: Gly = 2.0; Dab = 1.0.

2HCl·H-Gly-Gly-Dab Derivative of Poly(L-lysine). 100% Modified Polymer. Boc-Gly-Gly-Dab(Adoc)-OH (0.60 g, 1.18 mmol) was dissolved in 12 mL of anhydrous THF. To this solution, cooled to 0 °C, were added 0.16 g (1.41 mmol) of HONHS and 0.29 g (1.41 mmol) of DCCl. The mixture was left stirring at 0 °C for 1 h and at 4 °C for 2 days. The solid dicyclohexylurea was removed by filtration and washed with cold THF. The organic solution, containing Boc-Gly-Gly-Dab(Adoc)-OSu, concentrated to 8 mL, was added, slowly, under stirring, to an aqueous solution of poly(L-lysine) (0.587 mmol in 8 mL) containing 1.98 mL (1.98 mmol) of 1 N NaOH. The mixture was left stirring at 4 °C for 2 days and then evaporated. The solid residue was dissolved in 7 mL of 95% TFA (containing 5% water), and the solution was left stirring at room temperature for 2 h. The solvent was evaporated and the solid residue redissolved in water. After removal of the insoluble material by filtration, the clear solution was repeatedly dialyzed (cut-off 8000) vs. water and then vs. a solution of 10^{-3} M HCl and finally lyophilized: yield, 0.225 g; $[\alpha]^{20}_D = -45°$ (c = 1, water).

Amino acid ratio of the acid hydrolysate: Gly = 2.05 (2); Dab = 0.95 (1); Lys = 1.06 (1).

2HCl·H-Gly-Gly-Dab Derivative of Poly(L-lysine). 50% Modified Polymer. Boc-Gly-Gly-Dab(Adoc)-OH (0.34 g, 0.66 mmol) was dissolved in 11 mL of anhydrous THF. To this solution, cooled to 0 °C, were added 0.092 g (0.796 mmol) of HONHS and 0.164 g (0.796 mmol) of DCCl. The mixture was left stirring at 0 °C for 1 h and at 4 °C overnight. The solid dicyclohexylurea was removed by filtration and washed with cold THF (9 mL). The organic solution, containing Boc-Gly-Gly-Dab(Adoc)-OSu, was added slowly, under stirring, to an aqueous solution of poly(L-lysine) (0.78 mmol in 20 mL) containing 1.6 mL (1.6 mmol) of 1 N NaOH. The mixture was left at 4 °C, under stirring, for 2 days and finally evaporated. The solid residue was dissolved in 10 mL of 95% TFA, left stirring for 2 h, evaporated, and then redissolved in water. After removal of the insoluble material by filtration, the clear solution was repeatedly dialyzed (cut-off 8000) vs. water, and then vs. a solution of HCl 10^{-3} M and finally lyophilized: yield, 0.28 g; $[\alpha]^{20}_D = -56°$ (c = 1, water).

Amino acid ratio of the acid hydrolysate: Gly = 1.00; Dab = 0.50; Lys = 1.00.

Methods. Solvents were usually evaporated at 30–40 °C under reduced pressure.

Melting points were determined by a Tottoli apparatus or a Leitz Laborlux 12 apparatus, equipped with a Leitz 350 warming plate, and are uncorrected.

Optical rotations were determined at 20 °C with a Perkin Elmer 141 polarimeter.

Samples for elemental analyses were dried in vacuo over P_2O_5 at 40 °C.

Thin-layer chromatography (TLC) was carried out on silica gel 60 plates F-254 (Merck AG, Darmstadt, West Germany), using the following eluent systems: I, 1-butanol/acetic acid/water (3:1:1); II, 1-butanol/pyridine/acetic acid/water (60:40:12:48); III, chloroform/acetic acid/benzene (85:10:5). The amino acid derivatives and peptides were visualized by spraying the chromatograms with ninhydrin reagent[14] for the free amino groups and the modified chlorine reagent[15] for all peptide derivatives.

The acid hydrolyses were carried out in azeotropic hydrochloric acid for 22 h at 110 °C in sealed evacuated vials. The amino acid analyses were performed on a Carlo Erba 3A28 amino acid analyzer.

Aqueous solutions of $2CF_3COOH$·H-Gly-Gly-Dab-OH and of 2HCl·H-Gly-Gly-Dab derivatives of poly(L-lysine), for spectroscopic measurements, were prepared by dissolving a weighed amount of material in 0.01 M phosphate buffer or in distilled water. In the latter case, the pH was brought to the desired value by adding appropriate amounts of concentrated solutions of NaOH. Peptide concentrations were determined by weight and peptide content of the samples (as determined by quantitative amino acid analyses).

Aqueous solutions of Cu(II) and Ni(II) were prepared by dissolving a weighed amount of $CuCl_2$ and $Ni(ClO_4)_2$·$6H_2O$ in the minimum amount of 2 M HCl and then diluting the solutions to the final volume with 0.01 M HCl. Ion concentrations were precisely determined by atomic absorption spectroscopy, using a Perkin Elmer Model 360 AA spectrometer, equipped with multielement hallow cathode lamps. pH measurements were performed with a Methrom Herisau Model E 510 pH-meter, equipped with a glass microelectrode, calibrated with standard solutions. Absorption measurements were carried out with a Perkin Elmer Model 554 double-beam spectrophotometer. The optical path length ranged from 1.00 to 5.00 cm.

CD measurements were performed at 25 °C, using a Varian Cary 61 dichrograph in the visible absorption region and a Jasco Model J-500 automatic recording spectropolarimeter, equipped with a Jasco DP-501N data processor, in the UV region. With the Jasco instrument the signal-to-noise ratio was improved by accumulating 4–64 scans, depending upon the intensity of the CD spectrum. The Jasco dichrograph was equipped with a sample alternator, which allowed the recording of the base line immediately after each scan. Difference CD spectra were accumulated and stored in the computer, following the procedure previously described,[16] which eliminates errors caused by eventual drifts of the base line. The optical path length was usually 0.1 cm in the UV absorption region and 5.00 cm in the visible absorption region.

Additions and subtractions of the CD spectra were performed directly by the computer. The spectra reported in this paper are original CD curves. In the case of the modified polymers, the CD spectra in the peptide absorption region are reported in terms of molar ellipticity units *per mole of backbone peptide residue* ($[\vartheta]_R$), while in the visible region they are reported in terms of molar ellipticity units *per mole of tripeptide unit* ($[\vartheta]_{TRI}$). For the tripeptide, the spectra are always reported in terms of $[\vartheta]_{TRI}$.

Results and Discussion

Peptide Synthesis and Polymer Derivatization. The reaction scheme for the synthesis of the protected tripeptide and for its coupling to the polymeric matrix is shown in Figure 2. The protecting groups Boc and Adoc were selected because they can be simultaneously removed by dissolving the derivatized polymers in TFA. Derivatization of poly(L-lysine) was achieved by condensation of the N-protected tripeptide unit via *N*-hydroxysuccinimide active ester. The reaction is very fast and quantitative and polymeric adducts with various extents of side-chain

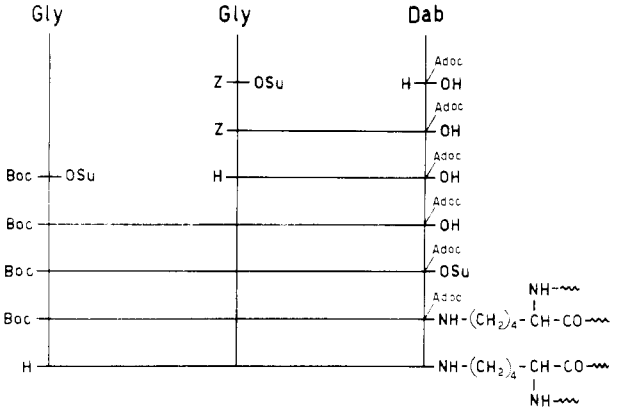

Figure 2. Reaction scheme for the synthesis of the protected tripeptide and for the coupling to poly(L-lysine).

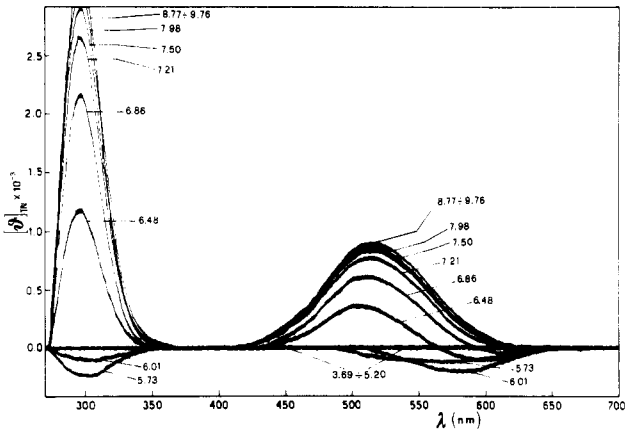

Figure 3. CD spectra in the visible–near-UV absorption region of H-Gly-Gly-Dab-OH in aqueous solution at various pH values (indicated on the spectra), at Cu(II)/tripeptide molar ratio R = 1.15. $[\vartheta]_{TRI}$ on the ordinate scale represents molar ellipticity units *per mole of tripeptide*. In all subsequent spectra CD spectra in the visible–near-UV absorption region are always reported in terms of $[\vartheta]_{TRI}$.

modification can be prepared by changing the relative ratios of N-protected tripeptide and poly(L-lysine). This coupling procedure in principle could lead to extensive racemization of the Dab residue.[17] This event would certainly affect the chiroptical properties of the Cu(II) complexes but should be without effect on the affinity of the derivatized polymers toward the metal ions. No specific racemization tests have been carried out on the derivatized polymers. However, we have observed (see next sections) that Cu(II) and Ni(II) complexes of the free tripeptide H-Gly-Gly-Dab-OH and of the tripeptide anchored to the polymeric matrix have comparable optical rotatory properties in the visible region. We therefore conclude that under our experimental conditions there was not extensive racemization during the final condensation process.

Cu(II) and Ni(II) Complexes of H-Gly-Gly-Dab-OH. Complex formation of the free tripeptide with Cu(II) and Ni(II) ions was preliminarily investigated by CD and absorption spectroscopy in the visible and in the far UV. The CD results obtained in the visible absorption region in aqueous solution at various pH values and in the presence of a stoichiometric excess of Cu(II) ions are reported in Figure 3. Complex formation is monitored by the appearance of optical activity in the d–d transition region of the metal ion. In the examined range of pH all spectra do not fit a single isodichroic point. The shape of the CD patterns is strongly pH-dependent. At pH 5.73 there are two weak negative bands, at 575 (very broad) and 300 nm.

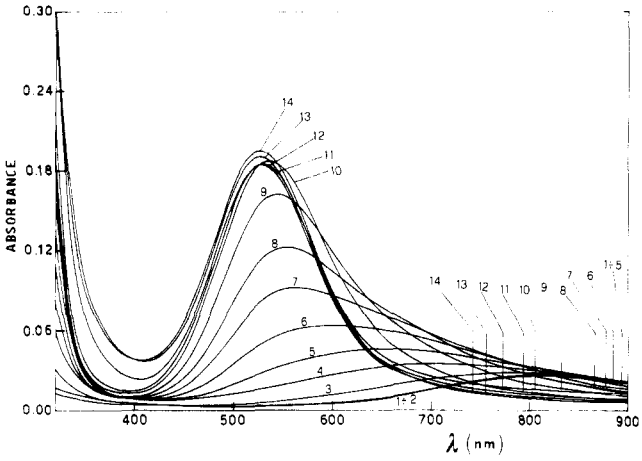

Figure 4. Absorption spectra of H-Gly-Gly-Dab-OH in aqueous solution at various pH values and in the presence of Cu(II) at R = 1.15. The tripeptide concentration was 3.66×10^{-4} M, in a 1-cm path length cell. The pH values of each curve are as follows: (1) 3.50; (2) 3.95; (3) 5.00; (4) 5.29; (5) 5.50; (6) 5.68; (7) 5.85; (8) 6.00; (9) 6.23; (10) 6.54; (11) 7.04; (12) 7.61; (13) 9.18; (14) 9.80.

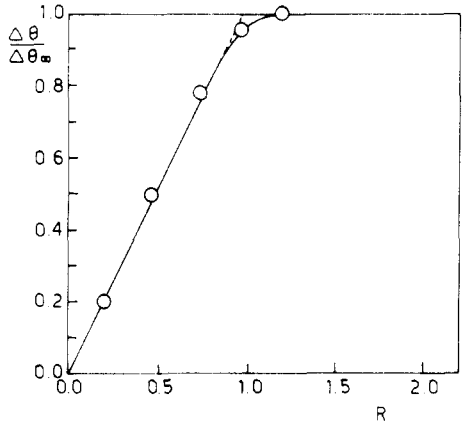

Figure 5. Relative variation of molar ellipticity of the free tripeptide at pH 7.50 as function of the molar ratio Cu(II)/tripeptide R.

At pH 6.01 the negative band at 575 nm becomes more intense, while that at 300 nm becomes weaker. A further increase of the pH to 6.48 induces the formation of a system of two oppositely signed bands, at 600 nm (negative) and 510 nm (positive), while the absorption at 300 nm becomes strong and positive. Finally, at pH 7.21 the negative band at 600 nm disappears. The CD pattern reaches its final form at pH 7.5 and is characterized by a positive, broad band at 515 nm ($[\vartheta]_{TRI}$ = 858 molar ellipticity units), and by a stronger, positive band at ~297 nm ($[\vartheta]_{TRI}$ = 3034 molar ellipticity units).

These spectral characteristics indicate that more than one Cu(II)–tripeptide complex is formed in the examined pH range. This conclusion is further substantiated by absorption measurements in the visible region (Figure 4). The spectra, recorded at various pH values, do not fit a single isosbestic point, thus confirming that more than one complex is formed.

The stoichiometry of the final complex at pH ≥ 7.5 is 1:1. This is clearly shown by the saturation curve reported in Figure 5. The relative variation of molar ellipticity at 515 nm as a function of the Cu(II)/tripeptide molar ratio R is linear and extrapolates to R = 1. The structure of this complex is different from that observed with the tripeptide Gly-Gly-His. In that case, in fact, a system of two oppositely signed CD bands, at 576 (negative) and 494 nm (positive), was observed at pH ≥ 5.50, with an additional

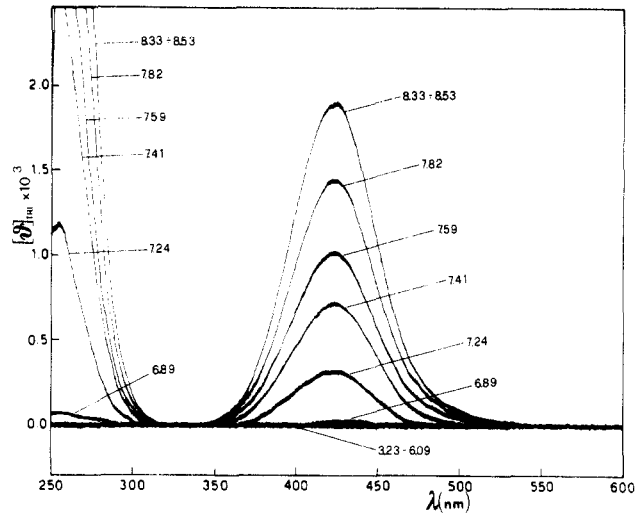

Figure 6. CD spectra in the visible–near-UV absorption region of H-Gly-Gly-Dab-OH in aqueous solution at various pH values (indicated on the spectra) and at Ni(II)/tripeptide molar ratio R = 1.13.

positive band at 310 nm. These features are consistent with the presence of deprotonated peptide nitrogens and of the imidazole nitrogen at the coordination sites of copper in a square planar geometry (Figure 1). With the tripeptide Gly-Gly-Dab a CD double band system in the Cu(II) d–d transition region can be observed only at pH 6.48. At higher pH there is a single strong CD absorption at 515 nm. We should therefore conclude that under these experimental conditions a structure such as that shown in Figure 1 with the γ-amino group replacing the imidazole nitrogen is not formed. On the other hand, the strong positive band at 300 nm indicates again the presence of deprotonated peptide nitrogens in the coordination sphere of Cu(II). The most likely structure, commonly observed in tripeptides, should involve the amino nitrogen, two deprotonated peptide nitrogens, and the carboxylate oxygen in a tetragonal arrangement around the ion.

Very similar results have been obtained with Ni(II) ions. The CD spectra recorded in presence of a stoichiometric excess of metal ions at various pH values are shown in Figure 6. In this case the increase of the pH causes the formation of a single CD band at 425 nm and of an additional CD band in the near UV at 260 nm. Consistent with literature results,[18] the entire spectrum is blue-shifted by ~90 nm with respect to that of the corresponding Cu(II) complex. Again the absence of two oppositely signed bands in the visible absorption region indicates that the γ-NH_2 group of Dab does not replace the imidazole nitrogen in the structure of Figure 1.

Conformational Properties of Derivatized Lysine Polymers. The CD spectra of the 100% and 50% modified lysine polymers at various pH values are reported in Figures 7 and 8. At acidic or neutral pH, both polymers exhibit the typical CD pattern of the random coil structure with a deep minimum at ~196 nm ($[\vartheta]_R \simeq$ 54 000 molar ellipticity units) and negative dichroism down to 185 nm. Increasing pH reveals different behavior in the two polymers. The 100% modified polymer is unable to assume a fully ordered conformation upon neutralization of charged, side-chain amino groups. The only noticeable effect of the pH increase is a reduced intensity of the negative CD band, which also shifts to the red, and the formation of a weak shoulder at ~220 nm. These features indicate that only a very small extent of ordered structure can be formed at alkaline pH. On the contrary, the 50% derivatized polymer at pH ≥ 11.59 exhibits the two

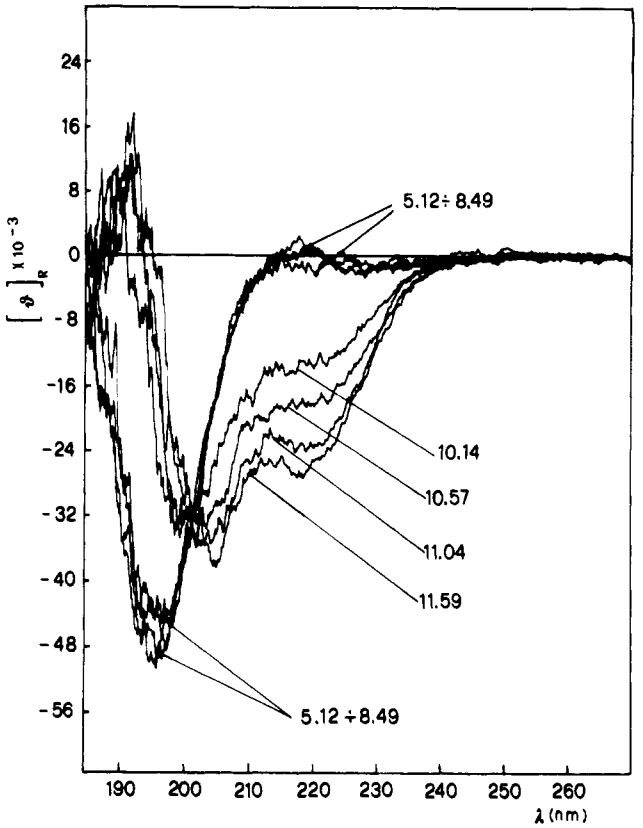

Figure 7. CD spectra in the far-UV absorption region of the 50% derivatized polymer in aqueous solution at various pH values (indicated on the spectra). $[\vartheta]_R$ indicates molar ellipticity units *per mole of backbone peptide residue.* In all subsequent figures, CD spectra in the far-UV are reported in terms of $[\vartheta]_R$.

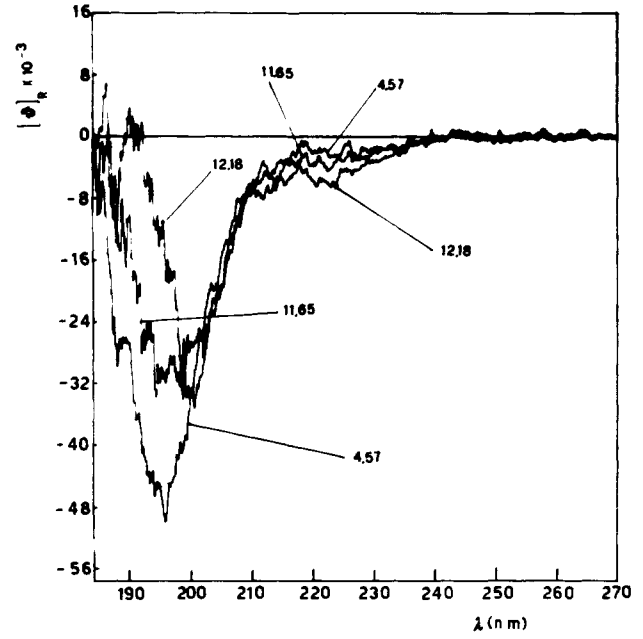

Figure 8. CD spectra in the far-UV absorption region of the 100% derivatized polymer in aqueous solution at various pH values (indicated on the spectra).

characteristic negative dichroic bands at 222 and 206 nm, diagnostic for the presence of the right-handed α-helical conformation. The intensities of the two bands (−26 000 and ~−34 000 ellipticity units) are slightly lower than those typical of the α-helix.[19] As discussed in our previous paper,[2] this effect should be attributed to the contribution of the optically active side-chains, very likely in an unordered structure.

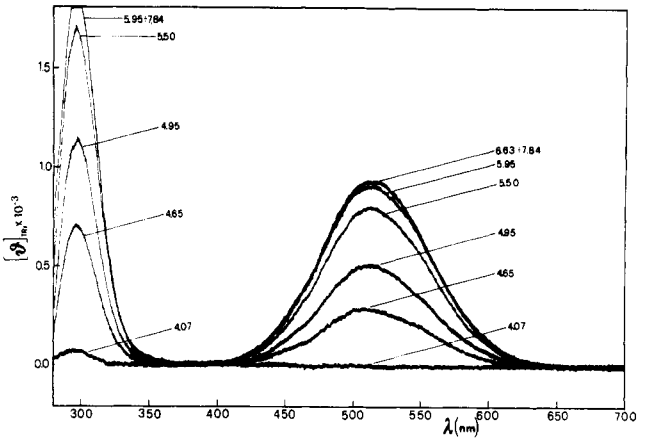

Figure 9. CD spectra in the visible–near-UV absorption region of the 100% derivatized polymer, in aqueous solution at various pH values (indicated on the spectra), and in the presence of an excess of Cu(II) ions [R = Cu(II)/tripeptide = 1.15].

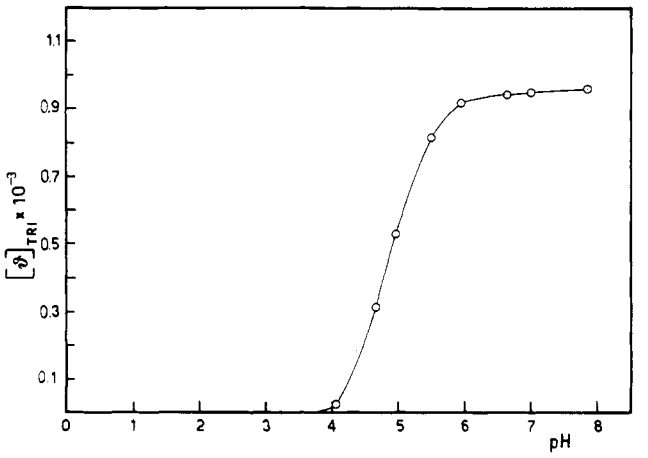

Figure 10. pH dependence of molar ellipticity at 515 nm for the 100% derivatized polymer at Cu(II)/peptide molar ratio 1.15.

Binding Properties toward Cu(II) and Ni(II) Ions of the Modified Lysine Polymers. The CD spectra in the visible absorption region of the 100% modified polymer in aqueous solution at various pH values and in the presence of a stoichiometric excess of Cu(II) ions are reported in Figure 9. In the examined pH range, a system of two oppositely signed CD bands in the Cu(II) d–d transition region was never observed. Complex formation causes in fact the appearance of a positive band in the visible at 515 nm and of an additional band, also positive in the near-UV, at 300 nm. The spectral change appears complete at pH ≥ 6.63. This is more evident from Figure 10, where the intensity of the CD band at 515 nm is plotted as a function of pH. The S-shaped profile reaches a plateau at pH ≥ 6.65.

Differently from what observed with the free tripeptide, only one complex species seems to be formed in the examined pH range. This conclusion follows from the results of Figure 8 and also from the absorption spectra in the visible region (Figure 11). The spectra recorded at different pH values and in presence of the stoichiometric amount of Cu(II) fit a well-defined isosbestic point at ~700 nm, indicating the presence of a two-component equilibrium system, namely free Cu(II) and a Cu(II)–polymer complex. The free tripeptide H-Gly-Gly-Dab-OH in the pH range 3–7.5 can form more than one complex because of the presence of the free, terminal carboxyl group, which can also be involved in the coordination to the metal ion, allowing the formation of different structures at different pH values. The complex formed by the free tripeptide at

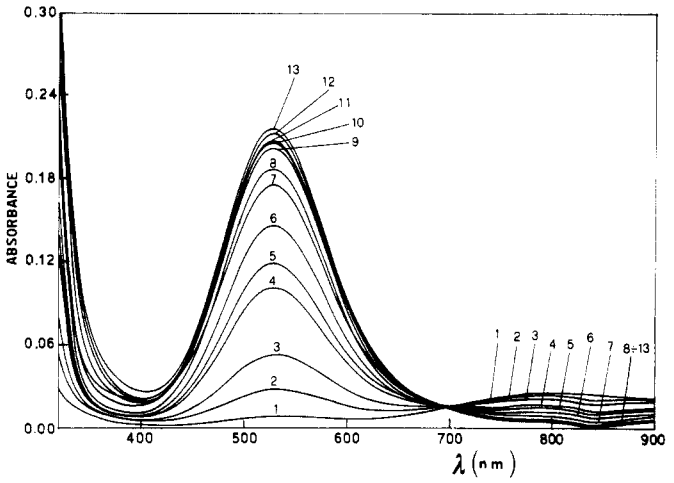

Figure 11. Absorption spectra in the visible absorption region of the 100% derivatized polymer in aqueous solution at various pH values and in the presence of the stoichiometric amount of Cu(II). The polymer concentration was 4.68×10^{-4} M residue in a 1-cm cell. The pH values of each curve are as follows: (1) 3.98; (2) 4.22; (3) 4.43; (4) 4.75; (5) 4.88; (6) 5.19; (7) 5.45; (8) 5.76; (9) 6.26; (10) 6.81; (11) 7.58; (12) 9.02; (13) 9.46.

pH 7.5 exhibits a CD pattern nearly identical with that of the tripeptide anchored to the polymeric matrix at pH $\geq$ 6.63. The comparable optical activity in the two cases also indicates that no extensive racemization took place in the coupling process of the chelating unit to the polymer. The stoichiometry of the Cu(II) complex formed at pH $\geq$ 6.63 is again 1:1 as with the tripeptide. A saturation curve identical with that shown in Figure 5 has been obtained, whose initial linear portion in fact extrapolates to $R = 1$.

Substantially identical results have been obtained with the 50% derivatized poly(L-lysine) sample. The CD spectra in the visible absorption region (not shown) of the polymer in aqueous solution at various pH values and in the presence of a stoichiometric excess of Cu(II) are identical with those reported in Figure 9. Also in this case the corresponding visible absorption spectra (not shown) fit a single isosbestic point, thus confirming the formation of only one complex species.

The binding properties toward Ni(II) ions of the derivatized polymers are very similar to those of the free tripeptide. The CD spectra in the visible absorption region of the 50% modified lysine polymer in aqueous solution at various pH values and in the presence of the stoichiometric amount of Ni(II) are shown in Figure 12. As in the case of the free tripeptide a positive band is observed at ~425 nm with an additional stronger positive band in the near UV at 260 nm. The only significant difference in the dichroic properties of the Ni(II) complex of the anchored tripeptide and of the free tripeptide is the presence of a shoulder at ~475 nm in the CD spectrum of the Ni(II)–polymer complex. Again this difference is probably due to the participation of the C-terminal carboxyl group of the free tripeptide in the coordination process.

Substantially identical results have been obtained with the 100% derivatized polymer in the presence of Ni(II) ions. The chiroptical properties (not shown) are identical with those of Figure 12.

The spectral characteristics of the Cu(II) complex of the tripeptide unit anchored to the polymeric matrix, in particular the absence of two oppositely signed CD bands in the d–d transition region of the metal ion, indicate that a structure such as shown in Figure 1 is not formed. In other words the γ-amino group of the Dab residue is not equivalent to an imidazole nitrogen of His in Cu(II) or

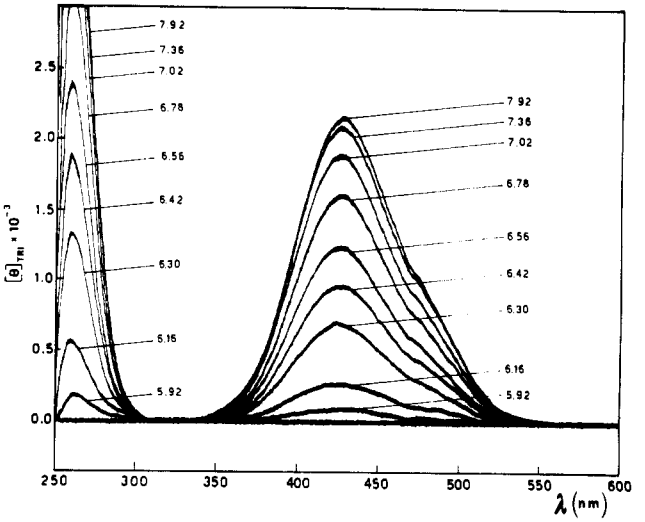

Figure 12. CD spectra in the visible–near-UV absorption region of the 50% derivatized polymer in aqueous solution at various pH values (indicated on the spectra) and in the presence of an excess of Ni(II) ions [R = Ni(II)/tripeptide = 1.1].

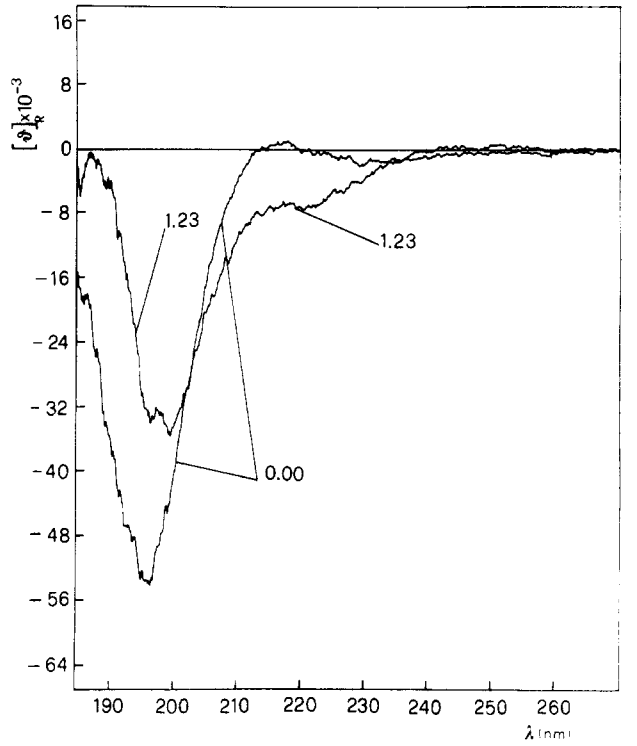

Figure 13. CD spectra in the far-UV absorption region of the 50% derivatized polymer in aqueous solution at pH 7.7 in absence of Cu(II) ions ($R = 0.0$) and in the presence of an excess of Cu(II) ions ($R = 1.23$).

Ni(II) coordination. The spectral characteristics suggest a structure involving the terminal amino group and the three consecutive deprotonated peptide nitrogens of the side-chains in a planar, tetragonal arrangement around the metal ion.

Effect of Ion Binding on Polypeptide Conformation. In order to determine the effects of ion binding at the side-chain tripeptide units on the conformation of the polypeptide backbone, CD measurements have been carried out in the far-UV absorption region. The CD spectra of the 50% derivatized polymer in aqueous solution at pH 7.75, in the absence of Cu(II) ions and in presence of an excess of Cu(II) ions, are reported in Figure 13. Complex formation causes a substantial decrease of the negative band at 197 nm, which also shifts to the red, and the

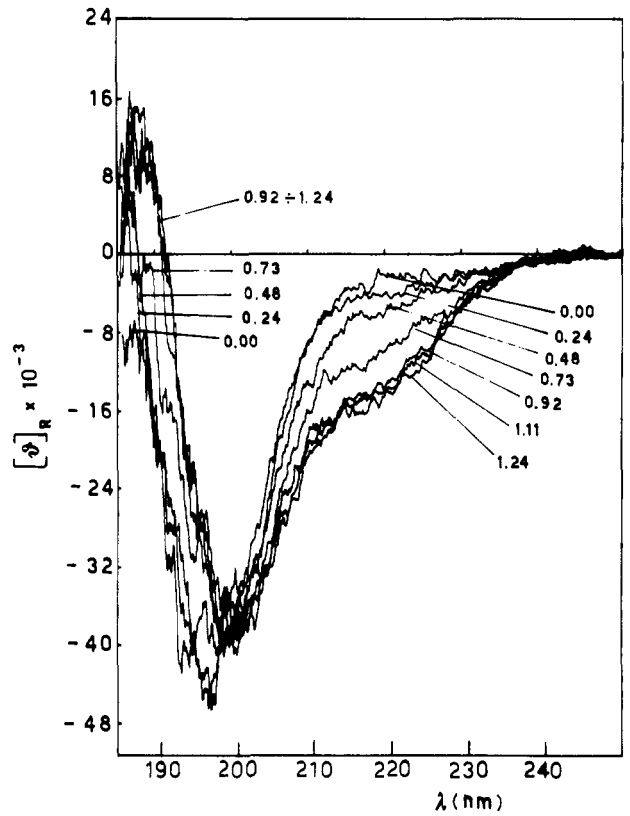

Figure 14. CD spectra in the far-UV absorption region of the 100% derivatized polymer in aqueous solution at pH 6.78 and in the presence of increasing amount of Cu(II). The molar ratios Cu(II)/tripeptide are indicated on the spectra.

appearance of a negative band at 220 nm. The spectral changes upon complex formation in the 100% derivatized polymer follow the same trend (Figure 14). Also in this case, a negative shoulder is formed around 220 nm, and there is a decrease and a red-shift of the negative band at 197 nm.

In principle these changes may reflect either a contribution to the optical activity from the complexed side chains or the formation of a certain amount of ordered structure of the polypeptide backbone. To clarify this point we have determined the effect of complex formation on the far-UV chiroptical properties of the free tripeptide H-Gly-Gly-Dab-OH as reference compound. The results, shown in Figure 15, clearly indicate that complex formation does not induce appreciable changes of the CD pattern at ~190 nm and above 215 nm, while a positive band forms at 205 nm. Thus the formation of a negative band at 220 nm upon Cu(II) binding must be entirely due to a conformational change of the peptide backbone. Actually from the results of Figures 13–15 the net variation of the CD spectrum consequent to the conformational change of the main chain can be separated from that of the side chains on the assumption that all contributions to the optical activity are additive. In the case of the 100% derivatized polymer, the difference between the spectra recorded at $R = 1.24$ and $R = 0$ of Figure 14 contains contributions both from the conformational change of the main chain and from complex formation in the side chains

$$(\vartheta)_{R=1.24} - (\vartheta)_{R=0} = \Delta\vartheta_{conf} + \Delta\vartheta_{TRI} \qquad (1)$$

where $\Delta\vartheta_{conf}$ represents the variation of the CD pattern due to the conformational change and $\Delta\vartheta_{TRI}$ represents the variation of the CD pattern due to complex formation in the side chains. The quantity $\Delta\vartheta_{TRI} = [(\vartheta)_{TRI-Cu} - (\vartheta)_{TRI}]$ can be directly determined from Figure 15 (difference between the spectra recorded at $R = 1.03$ and $R = 0$), and subtracted from 1. The resulting spectrum thus represents the net varation of the chiroptical properties relative to the conformational change of the peptide backbone only. The results of these calculations from the spectra of Figures 13–15 relative to the two derivatized polymers are reported in Figure 16. We stress the point that the spectra of Figure 16 represent the variation of the CD pattern consequent to the conformational change of the main chain only in the limits of our assumption, i.e., additivity of the various contributions to the optical activity. Within this assumption the shape of these spectra in both cases indicate that complex formation in the side chains causes partial folding of the polypeptide backbone into a right-handed α-helical structure. The result is rather surprising for the 100% derivatized sample. In fact in a

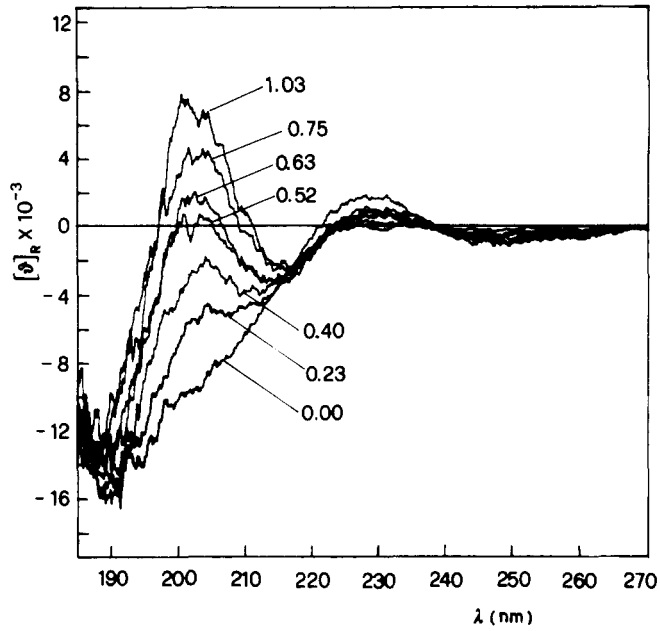

Figure 15. CD spectra in the far-UV absorption region of the tripeptide H-Gly-Gly-Dab-OH in aqueous solution at pH 7.5 and in the presence of increasing amounts of Cu(II) ions. The molar ratios Cu(II)/tripeptide are indicated on the spectra.

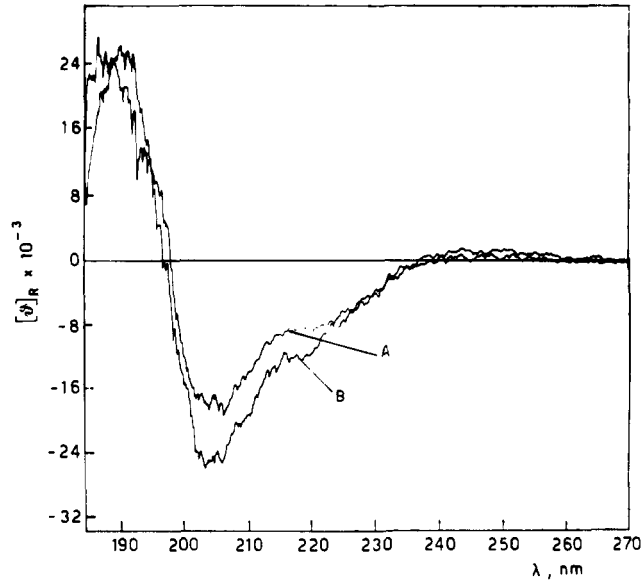

Figure 16. Net variation of the CD spectra in the far-UV absorption region relative to the conformational change of the peptide backbone of the 50% (spectrum A) and 100% (spectrum B) derivatized polymers calculated from Figures 15–17 according to eq 1.

previous section we have shown that this polymer is unable to fold into a α-helical conformation upon charge neutralization in the side chains. Folding occurs only upon complex formation.

Conclusions

The results presented in this work lead to the conclusion that the Dab residue at the third position in a peptide sequence cannot be considered equivalent to the His residue at the effect of Cu(II) and Ni(II) coordination. The spectroscopic characteristics of the metal complexes of the tripeptide, Gly-Gly-Dab either free or anchored to the polymeric matrix are completely different from those of the Cu(II) complexes of poly(L-Dab),[8] poly(L-histidine)[9] and of tripeptides containing His at the third position.[18] Thus a hexatomic chelate ring structure involving the γ-NH_2 group of Dab and the adjacent deprotonated nitrogen at two of the square-planar coordination sites of Cu(II) seems not to be formed at neutral pH. This conclusion rests mainly on the chiroptical properties of the complexes and, in particular, on the absence of two CD bands opposite in sign in the d–d transition of the metal ion.

As currently observed in tetrapeptides, the structure of the Cu(II) complexes of the side-chain tripeptide units should involve the terminal amino group and the three deprotonated peptide nitrogens at the coordination sites of the metal ion. In this case the arrangement should be tetragonal with the three chelate rings and the central metal ion on the same plane.

No attempt has been made to determine the relative stability of the Gly-Gly-Dab-Cu(II) complex formed at neutral pH. The stability of the Cu(II) complexes of peptides containing His at the third position is estimated to be $\sim 10^4$ times as high as that of other Cu(II)–tripeptide complexes.[20] It is therefore conceivable that the stability of the Gly-Gly-Dab–Cu(II) complex is lower than that of the corresponding Gly-Gly-His–Cu(II) complex. The observation that complex formation in the side chains induces folding of the peptide backbone in the α-helical conformation deserves a comment. The extent of folding increases with the degree of side-chain modification. This result has already been observed with 37% and 53% Gly-Gly-His-modified lysine polymers[2] and was tentatively explained with the reduced electrostatic repulsion among charged side chains, due to charge neutralization in the tripeptide units linked to Cu(II) ions. The results obtained with the 100% Gly-Gly-Dab-modified lysine polymer, described in the present work, reveal that an explanation based upon suppression of electrostatic repulsion is not sufficient. In fact complete charge neutralization, achieved in absence of Cu(II) ions at pH $\geq$ 12, does not induce folding of the peptide backbone into the α-helical conformation. Folding occurs only when the side-chain tripeptide units are involved in complex formation. This result suggests that some kind of interactions occur among complexed side chains. To understand the nature of these interactions further work is needed.

Acknowledgment. We are indebted to Dr. F. Magnarello and S. Da Rin Fioretto for skillful technical assistance. The work has been carried out with the financial support of the National Research Council (CNR) of Italy, Target oriented project "Chimica Fine e Secondaria".

Registry No. H-Dab-OH·2HCl, 6970-28-1; Adoc-F, 62087-82-5; $[Dab(Adoc)]_2Cu$, 100513-88-0; H-Dab(Adoc)-OH, 100513-79-9; Z-Gly-OSu, 2899-60-7; Z-Gly-Dab(Adoc)-OH, 100513-80-2; H-Gly-Dab(Adoc)-OH·CH_3COOH, 100513-82-4; Boc-Gly-Gly-Dab-(Adoc)-OH, 100513-83-5; H-Gly-Gly-Dab-OH·$2CF_3COOH$, 100513-85-7; Boc-Gly-Gly-Dab(Adoc)-OSu, 100513-86-8; poly(L-lysine) (homopolymer), 25104-18-1; poly(L-lysine) (SRU), 38000-06-5; Boc-Gly-OSu, 3392-07-2; Cu, 7440-50-8; Ni, 7440-02-0.

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