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COLLAGEN HELIX STABILIZATION BY HYDROXYPROLINE IN (ALA-HYP-GLY)$_n$

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SUMMARY (Ala-Hyp-Gly)$_n$ was synthesized and fractionated to yield three fractions in the molecular weight range 10,000 to 2,500. Optical rotatory and circular dichroism measurements indicated the collagen-like properties of this polymer in contrast to those reported for (Ala-Pro-Gly)$_n$. We conclude that hydroxyproline contributes to collagen-helix stability in general, independent of the presence of an adjacent proline residue as in previously studied synthetic polypeptides. (Pro-Hyp-Gly)$_n$ showed identical circular dichroism spectra whether polymerized via the nitrophenyl ester or tetraethylpyrophosphite.

An early proposal (1) that 4-hydroxyproline contributes to the stability of collagen was recently revived by demonstrating that (Pro-Hyp-Gly)$_n$ has greater thermal stability than (Pro-Pro-Gly)$_n$ (2) and by comparing unhydroxylated and hydroxylated αl chains of Type I collagen (3). Because the only model polypeptides studied contain the di-pyrrolidine sequences, -Pro-Pro- and Pro-Hyp-, it was of interest to examine the poly-tripeptide (Ala-Hyp-Gly)$_n$, especially since its unhydroxylated counterpart (Ala-Pro-Gly)$_n$ has been reported (4,5) to lack collagen-like structure. Our findings show that 4-Hyp indeed contributes to stability and collagen-like optical properties even when an adjacent Pro residue is absent. In addition, we describe optical properties of (Pro-Hyp-Gly)$_n$ made via the p-nitrophenyl ester as well as via tetraethylpyrophosphite.

MATERIALS AND METHODS

Commercial preparations included carbobenzoxy chloride, Z-Ala-OH* Z-Pro-OH, H-Gly-OEt,HCl, Z-Gly-ONp, and Z-Pro-ONp, all from Sigma Chemical Co. Tetraethyl-pyrophosphite was purchased from Aldrich Chemical Co., HBr in HOAc (30% w/v) from Eastman Kodak. HBr gas was purchased from Matheson Gas Products. Thin-layer chromatography and amino acid analysis were carried out by methods referred to earlier (6); designation of tlc solvents are those used earlier (6). Molecular weight of polypeptides was determined by a calibrated agarose A 1.5 column (7). Polypeptides were further fractionated by pooling selected effluent regions from the A 1.5 agarose column (7), passing these through a Biorad P-2 column to remove salts, lyophilizing, and rerunning

*Abbreviations of amino acids, peptides, N- or C-blocking groups are those listed in Biochem. J. 131, 1 (1973) or in J. Biol. Chem. 247, 977 (1971). Other abbreviations: tlc = thin layer chromatography; DCC = dicyclohexylcarbodiimide; TEA = triethylamine; HOAc = acetic acid, DMSO = dimethylsulfoxide; TEPP = tetraethylpyrophosphite.

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H-Hyp-Gly-ONp, HBr (10)
↓ Z-Ala-OH/DCC
Z-Ala-Hyp-Gly-ONp
↓ HBr gas
H-Ala-Hyp-Gly-ONp, HBr
↓ DMSO/TEA
$(Ala\text{-}Hyp\text{-}Gly)_n$

Scheme 1

Z-Pro-Hyp-Gly-OH (6)
↓ HBr/HOAc
H-Pro-Hyp-Gly-OH, HBr
↓ TEPP/pyridine
$(Pro\text{-}Hyp\text{-}Gly)_n$

Scheme 2

a small aliquot through the calibrated column to establish a molecular weight range for the fraction. Polypeptide concentration in solution was determined by measurement of hydroxyproline (8) in hydrolyzates. Melting points (Fisher-Johns hot stage) are uncorrected. CD spectra were obtained with a Jasco Model 20 spectropolarimeter using 1 mm jacketed cells with quartz windows. Thermal transition curves were obtained with a 1 cm jacketed cell; a temperature probe was inserted in an identical cell (dummy cell), similarly jacketed and thermostatted in series with the test cell, and plugged into a Digitac 5810 Thermometer (United Systems Corp., Dayton, Ohio). Before thermal transition readings, the polypeptide solution was stored at 5° for 10 days. During readings, temperature was raised stepwise from the lowest value, allowing 30 min at each temperature for equilibration.

RESULTS AND DISCUSSION

Synthesis of Polytripeptides $(Aly\text{-}Hyp\text{-}Gly)_n$ was prepared according to Scheme 1. To our knowledge, this tripeptide has not been previously polymerized, although the polymer $(Gly\text{-}Ala\text{-}Hyp)_n$ has been reported (9). $(Pro\text{-}Hyp\text{-}Gly)_n$ was prepared via the ONp ester as described earlier (10). An attempted simplification of this procedure -- by condensing Z-Pro-Hyp-OH directly with H-Gly-ONp — failed, although Z-Pro-Pro-OH has been successfully condensed with H-Gly-ONp (11). Scheme 2 outlines an alternative route to this polymer using TEPP. An attempt to polymerize H-Hyp-Gly-Pro-ONp,HBr, made by condensing Z-Hyp-Gly-OH (6) with Pro-ONp,HBr, yielded only low molecular weight material.

$(Ala\text{-}Hyp\text{-}Gly)_n$ Unfractionated preparations of $(Ala\text{-}Hyp\text{-}Gly)_n$ (average molecular weight 2500) showed no helical structure at 25° since optical rotation (589 nm) was unchanged by prior heating at 70° for 15 min or by addition of formic acid to 20%. In contrast, Fig. 1 shows temperature dependence of $[\alpha]$ for fractions of different molecular weight ranges. The lowest molecular weight fraction shows an almost linear decrease in $[\alpha]$ with increasing temperature, expected for a random coil (12). The higher fractions,

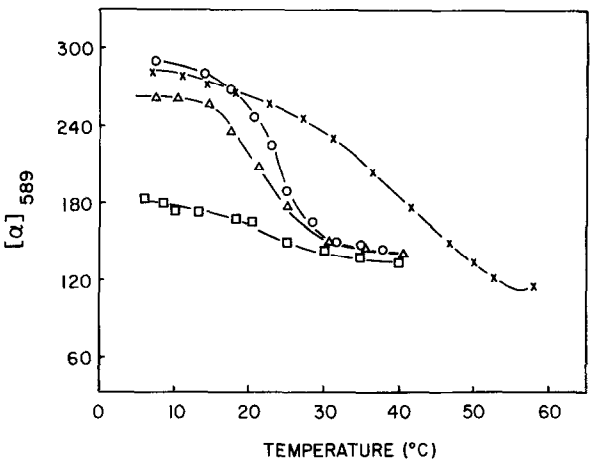

FIG 1. TEMPERATURE DEPENDENCE OF SPECIFIC ROTATION FOR $(ALA\text{-}HYP\text{-}GLY)_n$ OF DIFFERENT MOLECULAR WEIGHT FRACTIONS

10,000-7500 fraction —o—o—, 6500-4500 fraction —Δ—Δ—, 4100-2500 fraction —□—□—. Solvent was aqueous 0.1 M NaCl, except for —x—x— (6500-4500 fraction, run in 1,3-propanediol). In all cases, polypeptide concentration was 1.2 to 1.5 mg/ml.

however, have relatively higher [α] and exhibit typical helix-coil transitions, the transition temperature, $T_{\frac{1}{2}}$, increasing with average molecular weight. At 40°, as expected for disordered polypeptide chains, all three fractions have similar [α]. Similar sigmoidal transition curves were reported for $(Pro\text{-}Hyp\text{-}Gly)_{10}$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ (2).

Fig. 1 also shows the transition curve of $(Ala\text{-}Hyp\text{-}Gly)_n$ (6500 - 4500 fraction) in 1,3-propanediol. In this solvent, $T_{\frac{1}{2}}$ is shifted upward, suggesting that the helical structure is more stable in this weak hydrogen-bonding solvent than in water, a finding consistent with the conclusion of Brown et al. (13) from studies of the CD spectrum of $(Pro\text{-}Ser\text{-}Gly)_n$ in water and in 1,3-propanediol.

Fig. 2 shows CD spectra for the helical (7°) and random coil (40°) forms of $(Ala\text{-}Hyp\text{-}Gly)_n$ (10,000 - 7500 range), compared with those for $Pro\text{-}Hyp\text{-}Gly)_n$ and $(Ala\text{-}Pro\text{-}Gly)_n$ at 24°. Collagen-like properties for $(Ala\text{-}Hyp\text{-}Gly)_n$ are the positive band at 220 - 222 nm ([θ] = 4000 deg cm^2 $decimole^{-1}$), the negative band at 194 nm ([θ] = 33,000 deg cm^2 $decimole^{-1}$), and the disappearance or dimunution of these bands at 40°. Similar

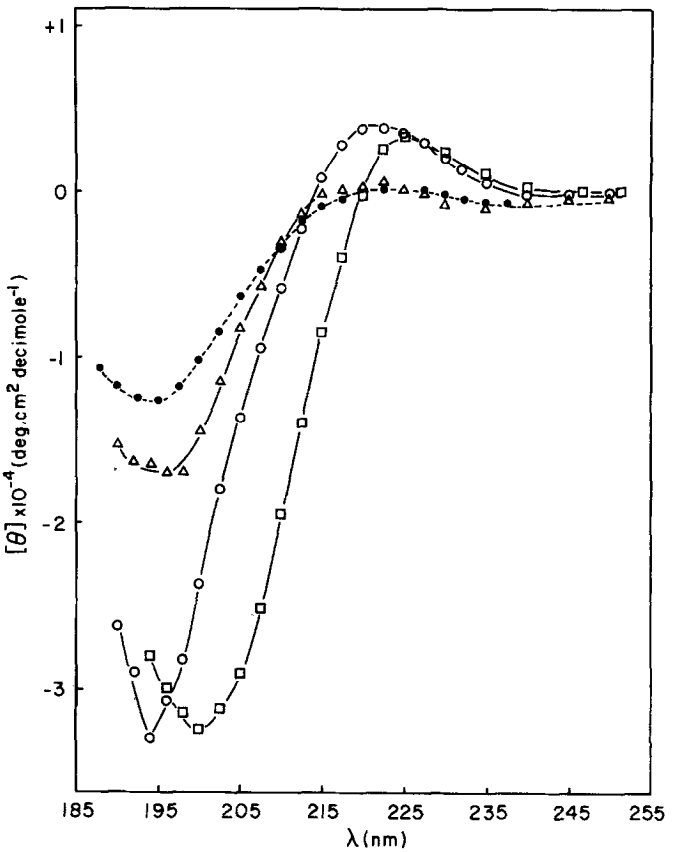

FIG. 2. CIRCULAR DICHROISM SPECTRA OF DIFFERENT POLYPEPTIDES

$(Ala\text{-}Hyp\text{-}Gly)_n$ (10,000-7500 molecular weight fraction) at 7° —o—o—, at 40° —Δ—Δ—. $(Pro\text{-}Hyp\text{-}Gly)_n$ (5500-4500 molecular weight fraction) at 24° —□—□—. $(Ala\text{-}Pro\text{-}Gly)_n$ (molecular weight 5000) —●—●—●—: data taken from Doyle et al. (5). Solvent was 0.01 M aqueous NaCl; [θ] is mean residue ellipticity.

CD changes with temperature were reported for rat skin collagen (14). It is noteworthy that at 40°, the CD spectrum of $(Ala\text{-}Hyp\text{-}Gly)_n$ resembles that of the non-helical $(Ala\text{-}Pro\text{-}Gly)_n$ at 24°. The reliability of our data for $(Ala\text{-}Hyp\text{-}Gly)_n$ is shown by the identity of the CD spectrum of $(Pro\text{-}Hyp\text{-}Gly)_n$ with that of $(Pro\text{-}Hyp\text{-}Gly)_{10}$, as earlier presented (15). Although both $(Ala\text{-}Hyp\text{-}Gly)_n$ and $(Pro\text{-}Hyp\text{-}Gly)_{10}$ form collagen-like helices and show qualitatively similar CD spectra, the CD bands are not expected at the same wavelengths since this depends on the content of secondary amino acids.

We conclude from these findings that the Hyp residue unquestionably stabilizes a collagen-like helix in $(Ala\text{-}Hyp\text{-}Gly)_n$, as it does in the dipyrrolidine model $(Pro\text{-}Hyp\text{-}Gly)_n$. Several proposals have been made to explain this stabilization. One, that postulates a cis-peptide bond between Gly and Pro in $(Pro\text{-}Hyp\text{-}Gly)_n$ (16), cannot apply to our model. Our data do not distinguish between the other two proposals, which postulate that the hydroxyl group of hydroxyproline forms an additional hydrogen bond, via a water molecule, either between two polypeptide chains (17) or within the same chain (18) in the collagen triple helix.

$(Pro\text{-}Hyp\text{-}Gly)_n$ The random polymer $(Pro\text{-}Gly\text{-}Pro)_n$, polymerized with TEPP (19), failed to show the clear-cut thermal transitions of $(Pro\text{-}Pro\text{-}Gly)_{10}$ (20). This difference could be attributed to the homogeneity of the latter polymer, even though the average molecular weights of both random and homogenous polymers were similar. Another possibility is incorporation of P-containing fragments in the polypeptide chain (19) formed by the TEPP method. For this reason, we compared $(Pro\text{-}Hyp\text{-}Gly)_n$ (refractionated, average molecular weight 5000) made via the ONp ester (10), with $(Pro\text{-}Hyp\text{-}Gly)_n$ (refractionated, average molecular weight 2700) made via TEPP according to Scheme 2. Both polymers showed essentially identical CD spectra, represented by that for $(Pro\text{-}Hyp\text{-}Gly)_n$ in Fig. 2. Our data, therefore, fail to show interference with helix formation attributable to TEPP-polymerization.

It is of interest to note that, in our hands, several preparations of $(Pro\text{-}Hyp\text{-}Gly)_n$ by the method of DeTar et al. (10) consistently yielded an average polymer size of 2000 - 3000, in contrast to the higher molecular weights reported earlier (10). The difference may be due to our method of molecular weight estimation in 1M $CaCl_2$ (7), designed to prevent peptide chain aggregation.

EXPERIMENTAL

These descriptions are limited to new compounds or new methods of preparation.

H-Pro-Hyp-Gly-OH,HBr Z-Pro-Hyp-Gly-OH (6) (0.9 g) was dissolved in 1 ml of HOAc and treated with 6 ml of HBr/HOAc. After 1 hour, the hydrobromide was precipitated by addition of 70 ml of dry ether. Crystals were washed with ether, redissolved in 20 ml of isopropanol and reprecipitated with ether. Yield, 0.7 g (89%); tlc, single spot by charring or ninhydrin (solvent C, R_f 0.29; solvent G, R_f 0.17; solvent L, R_f 0.04).

(Pro-Hyp-Gly)$_n$ (Scheme 2) H-Pro-Hyp-Gly-OH,HBr (400 mg) was dissolved in 1 ml of pyridine, treated with 0.5 ml of TEPP and kept at 25°. After 2 days, the viscous solution was stirred with 5 ml of dry ether to yield a tan powder (465 mg). On agarose chromatography, a major peak was eluted at a position corresponding to molecular weight 1700. The leading third of this peak, when rechromatographed, gave a number-average molecular weight of 2700. Amino acid analysis of a hydrolyzate gave equimolar glycine, proline and 4-hydroxyproline.

Z-Hyp-Gly-Pro-ONp Z-Hyp-Gly-OH (6) (4.14 g) was suspended in 15 ml of acetonitrile and treated with 2 ml of TEA. H-Pro-ONp,HBr (prepared from Z-Pro-ONp) (4.12 g) was suspended in 40 ml of acetonitrile, stirred magnetically and treated with DCC (3 g). At 5°, the suspension of H-Pro-ONp,HBr/DCC was added dropwise (1 hour) to the stirred suspension of Z-Hyp-Gly-OH. After 5 hours, presumptive dicyclohexylurea was removed by filtration, the filtrate concentrated to 24 ml and added to 150 ml of 0.01 N HCl at 5°. The mixture was stirred overnight at 5°; the oily residue was dissolved in minimum ethyl acetate and dried to a yellow powder. Yield 5.2 g (75%); tlc (solvent B) gave a streak centered at Rf 0.4.

H-Hyp-Gly-Pro-ONp,HBr Z-Hyp-Gly-Pro-ONp (1 g) was dissolved in 1.5 ml of HOAc and treated with 7 ml of HBr/HOAc. After 1 hour, 75 ml of dry ether was added. The resulting powder was washed with ether and stored under vacuum.

(Hyp-Gly-Pro)$_n$ H-Hyp-Gly-Pro-ONp,HBr (0.5 g) was dissolved in 0.6 ml of DMSO and treated with 0.15 ml of TEA with stirring. After 3 days, the solution was treated with dry ether to yield 300 mg of a brown powder. A 50 mg sample, run on the calibrated agarose column, gave a symmetrical peak whose position corresponded to molecular weight less than 1000.

Z-Ala-Hyp-Gly-ONp H-Hyp-Gly-ONp,HBr (10) (3 g) was suspended in 10 ml of acetonitrile and treated with 1.6 g of DCC. To this solution at 5° was added dropwise (15 min) a 10 ml solution of acetonitrile containing 1.72 g of Z-Ala-OH and 1.07 ml of TEA. After stirring for 2 hours at 5° and 2 hours at 25°, dicyclohexylurea was removed by filtration and the filtrate evaporated to dryness in vacuo. The solid residue was stirred with 40 ml of ethyl acetate and filtered. Ether (80 ml) was added; the crystalline precipitate that formed weighed 1.2 g and gave 3 spots on tlc; the major spot (solvent A, R_f 0.83) was thought to be the desired product. Attempts at recrystallization failed, but further treatment of the ethyl acetate/ether filtrate with ether yielded crystals (0.8 g), mp 135-137°, single spot on tlc (solvent A, R_f 0.83). Calculated: C, 56.0%; H, 5.2%; N, 10.9%. Found: C, 55.9%; H, 5.3%; N, 11.3%. $[\alpha]$ (c = 0.1, MeOH) -84.1°.

H-Ala-Hyp-Gly-ONp,HBr The Z-tripeptide above (0.7 g) was dissolved in 5 ml of CH_2Cl_2/2.5 ml of trifluoracetic acid, and treated for 1 hour with gaseous HBr. The reaction mixture was poured into 20 ml of dry ether, the precipitate was washed with more ether and dried under vacuum, yielding 0.6 g of powder which foamed on heating and gave no true mp. Further stirring in 10 ml of hot ethyl acetate yielded a filtrable powder, giving a single major spot on tlc (solvent A, R_f 0.2).

(Ala-Hyp-Gly)$_n$ 0.42 g of the above hydrobromide was suspended in 8 ml of DMSO, treated with 0.13 ml of TEA and stirred for 4 days. DMSO was removed by lyophilization and the residual solid was washed twice with 15 ml of ether. The dried powder was suspended in 20 ml of water and dialyzed at 5° for 3 days against 1 liter of water (3 changes). The retentate was lyophilized, yielding 83 mg of powder. Average molecular weight of unfractionated material was 2500. Amino acid analysis of a hydrolyzate gave equimolar alanine, 4-hydroxyproline and glycine.

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