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Conformational Studies of the Human Complex-Forming Glycoprotein, Heterogeneous in Charge: Protein HC†

José G. Gavilanes, Carlos López-Otín, Francisco Gavilanes, and Enrique Méndez*

ABSTRACT: Human complex-forming glycoprotein, heterogeneous in charge, is a single polypeptide chain widely distributed in physiological fluids. The conformation of the protein has been studied with attention to the secondary and tertiary structures. Circular dichroism and predictive methods from the amino acid sequence have been employed for the characterization of the secondary structure. This is composed of 20% α-helix, 21% β-structure, 29% β-turns, 30% aperiodic conformation, and an average number of residues per helical segment of nine. Titration of the protein indicated the existence of two groups for the tyrosine residues, each of them composed of three and five residues. The four tryptophan residues of the molecule are located in two different polarity microenvironments, according to the fluorescence studies. These observations are corroborated by studying the hydropathic profile of the protein. From this study, three different domains are observed in the protein, one of them being exposed and containing the main part of the unordered structure of the molecule. The chromophore naturally associated with the protein has been resolved in three fluorescent units not dependent on the protein conformation. These bands have been observed centered around 290, 360, and 410 nm, which do not correspond to any described chromophore.

Human complex-forming glycoprotein, heterogeneous in charge (protein HC), is a recently described low molecular weight glycoprotein originally isolated from normal human urine (Tejler & Grubb, 1976). It shows a considerable charge heterogeneity, carries an unidentified yellow-brown chromophore, and has been immunochemically demonstrated to occur in normal human plasma where a considerable part of the immunoreactivity is complexed with IgA (immunoglobulin A) (Tejler & Grubb, 1976). Protein HC displays appreciable charge heterogeneity on agarose gel electrophoresis and on isoelectric focusing, which does not diminish after desialylation (Tejler & Grubb, 1976). The complete amino acid sequence of protein HC isolated from the urine of an individual was reported in a preliminary paper (López et al., 1981). Further studies revealed the presence of an additional tryptophan residue at position 36 (C. López et al., unpublished results). No evidence for sequence variability of the single polypeptide chain of protein HC was found. However, protein HC isolated from a pool of urine from several individuals contains molecules with different COOH-terminal amino acid sequences (López et al., 1982). Computer analysis according to Dayfoff's program (Dayhoff, 1978) did not reveal any significant homology to any known protein. The protein is immunochemically and physicochemically related with two other recently described glycoproteins, α_1-microglobulin (Ekström et al., 1975; Ekström & Berggard, 1977; Svensson & Ravnskov, 1976; Takagi et al., 1979; Bernier et al., 1980) and α_1-microglycoprotein (Seon & Pressman, 1978), both isolated from the urine of patients with renal tubular dysfunction. Protein HC has been reported to be associated with lymphocyte cell surfaces (Tejler et al., 1976; Pearlstein et al., 1977) and to possess immunoregulatory properties (Logdberg & Akerstrom, 1981).

In this paper, we report the study of the secondary and tertiary structures of protein HC based on spectroscopic characterizations of the molecule and on the amino acid se-

† From the Department of Biochemistry, Faculty of Sciences, Complutense University, Madrid 3, Spain (J.G.G. and F.G.), and the Servicio de Endocrinología, Centro Ramón y Cajal, Carretera de Colmenar, Madrid 34, Spain (C.L.-O. and E.M.). *Received June 29, 1983.*

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quence recently reported (López et al., 1981), attempting to know the relationship between structure and function for this protein. In addition, the fluorescence of the chromophore associated with protein HC has been also studied in an effort to elucidate its structure and its possible contribution to the charge heterogeneity of protein HC.

Materials and Methods

Materials. Human protein HC has been purified from the urine of a single individual (J.L.) suffering from tubular proteinuria, as previously described (Tejler & Grubb, 1976). A single protein preparation has been employed for both spectroscopic and amino acid sequence studies.

Absorbance Measurements. Spectrophotometric determinations were carried out in a Cary 118 spectrophotometer at a scanning speed of 0.2 nm/s. Protein solutions were filtered through a Millipore filter (0.5 μm pore diameter) prior to the spectroscopic determination, which was performed at 20 °C in 1-cm optical-path cells.

Circular Dichroism Spectra. Circular dichroism (CD) spectra were measured in a Jobin Yvon Mark III dichrograph fitted with a 250-W xenon lamp at 0.2 nm/s scanning speed. Millipore- (0.5-μm pore diameter) filtered solutions of protein were studied in 0.05-cm optical-path cells in the far-UV region and 1-cm cells in the near-UV region. Concentration of the samples was determined by amino acid analysis. Circular dichroism results are the mean values of at least five determinations and are reported in terms of θ_{mrw}, mean residue ellipticities, in units of deg cm^2 dmol^{-1}. These values were calculated on the basis of 113 as the mean residue weight in protein HC.

Fluorescence Spectroscopy. The fluorescence spectra of protein HC have been performed on a Perkin-Elmer MPF-44E spectrofluorometer at 20 °C, in 1-cm optical-path cells. Excitation and emission spectra were obtained at least 3 times and were corrected for both detector response and light source. Protein solutions were filtered through a Millipore filter (0.5 μm pore diameter) prior to the spectroscopic determination. For emission spectra, 6- and 4-nm slit widths were employed for the excitation and emission beams, respectively, whereas 4- and 6-nm slit widths were used for excitation spectra.

Analytical Procedures. Protein hydrolyses were carried out at 110 °C for 20 h with 0.2 mL of 6 N HCl containing 0.05% 2-mercaptoethanol, in evacuated and sealed tubes. The analyses were performed in a Beckman 121 MB analyzer equipped with a Beckman integrator 126 data system. pH measurements were performed on a Radiometer titrator.

Prediction of the secondary structure of protein HC has been performed according to Chou & Fassman (1974a,b, 1978) on the basis of the amino acid sequence of the polypeptide chain. Tetrapeptides exhibiting average β-turn potential higher than 1.00, as well as higher than the corresponding value for α-helix and β-structure, and satisfying $f_i f_{i+1} f_{i+2} f_{i+3} > 0.75 \times 10^{-4}$, f_i being the β-turn frequency values, were assigned as β-turns. α-Helical and β-structure regions were determined according to the rules proposed by Chou and Fassman. Boundary residues in such regions were defined by considering the respective frequency values (Chou & Fassman, 1974a).

Results

Far-UV Circular Dichroism Study of Protein HC. The CD spectrum in the far-UV region for protein HC from a single individual is given in Figure 1. The protein has an extreme value centered at 213 nm, $\theta_{mrw} = -4400$ deg cm^2 dmol^{-1}. The ellipticity value at 222 nm is −2900 deg cm^2 dmol^{-1}. The shape of the spectrum as well as the low θ_{mrw} values would indicate

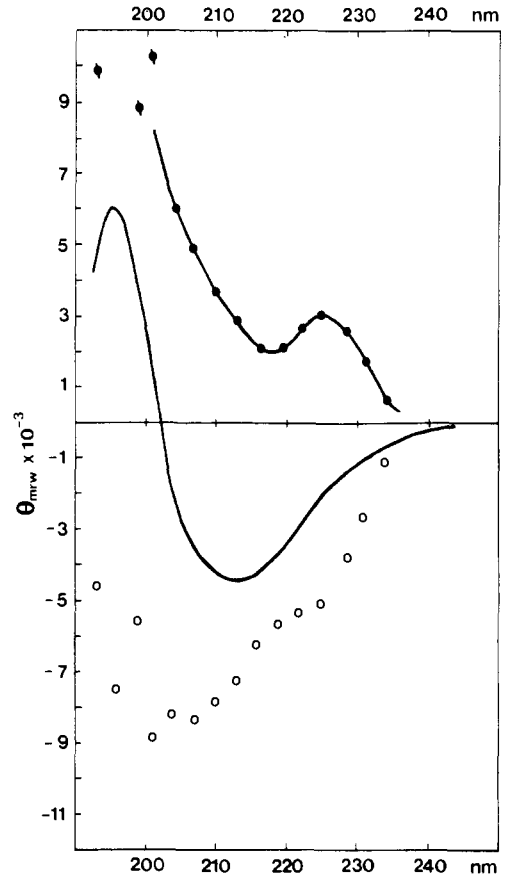

FIGURE 1: Circular dichroism spectrum of protein HC in the far-UV (—). The protein was dissolved in 0.1 M sodium phosphate buffer, pH 6.5. Circles correspond to the ellipticity values obtained for a secondary structure composed of 20% α-helix, 21% β-structure, 59% aperiodic conformation, and an average number of residues per helical segment of 9, according to the reference parameters of Chen et al. (1974). (●) Nonpeptide and specific β-turn contributions to the CD spectrum calculated as experimental minus theoretical values (see Results).

a nonsignificant α-helix content. However, the secondary structure estimation of protein HC, according to the reference parameters of Chen et al. (1974), does not give any coherent computer results to fit this experimental spectrum. On the other hand, protein HC is a glycoprotein with at least two Asn residues involved in N-glycosidic bonds to the carbohydrate moiety, residues located at positions 17 and 95 of the polypeptide chain (López et al., 1981). Also, this protein has an important content of carbohydrates, about 20% by weight. These facts, as well as both the presence of a yellow-brown chromophore in the protein and the reactivity of the –SH group of the cysteine residue at position 34 of the polypeptide chain of protein HC (Méndez et al., 1982), would explain the impossibility of any fit between the experimental spectrum and those obtained from protein reference values.

Secondary Structure Prediction of Protein HC. Taking into account those potential contributions to the CD spectrum, a prediction of the secondary structure of protein HC has been performed, on the basis of the amino acid sequence data (López et al., 1981), according to the procedure of Chou & Fassman (1974a,b, 1978). The result of this prediction is given in Figure 2 superimposed to the primary structure of the protein. From this study, protein HC would be composed of 20% α-helix, 21% β-structure, 59% aperiodic conformation, including β-turns, and an average number of residues per helical segment of 9. By use of these results, the theoretical spectrum of the protein has been calculated according to Chen et al. (1974) (Figure 1). Considering both experimental and

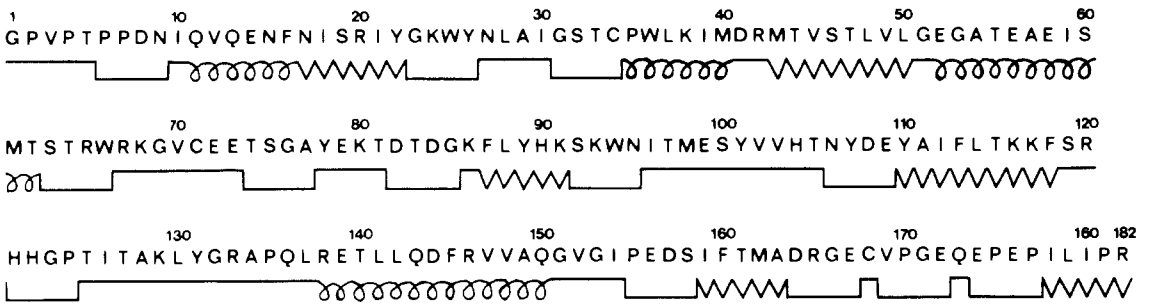

FIGURE 2: Secondary structure prediction for protein HC based on the amino acid sequence of the polypeptide, according to Chou & Fassman (1974a,b, 1978): (—) aperiodic; (lowered line) β-turns; (wavy line) β-structure; (looped line) α-helix.

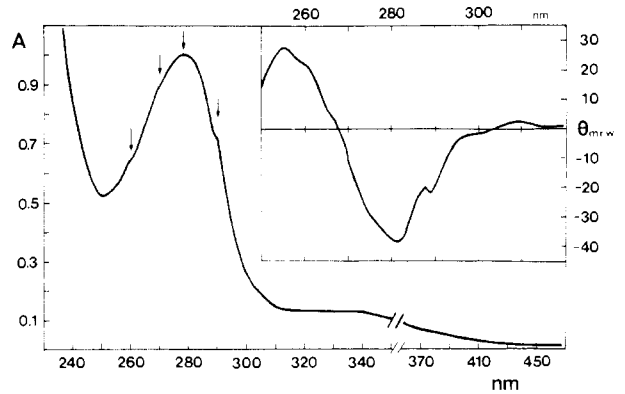

FIGURE 3: Absorption spectrum of protein HC in 0.1 M sodium phosphate buffer, pH 6.5, at 0.6 mg/mL polypeptide concentration. Arrows indicate the position of the maximum and shoulders in the spectrum. (Inset) CD spectrum of protein HC in the near-UV region. Protein was dissolved in the phosphate buffer, pH 6.5.

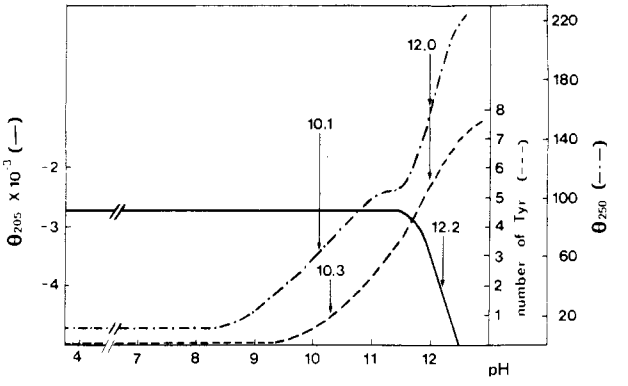

FIGURE 4: Results of titration of protein HC. Sample was dissolved in glass-distilled water and adjusted to each pH by the addition of suitable aliquots of NaOH. (--) Number of titrated tyrosine residues calculated from the A_{295} on the basis of an extinction coefficient at 295 for tyrosine of 2488 M^{-1} cm^{-1} (Wetlaufer et al., 1958). (—) Ellipticity at 205 nm. (-·-) Ellipticity at 250 nm. Arrows and numbers indicate the respective pK values observed.

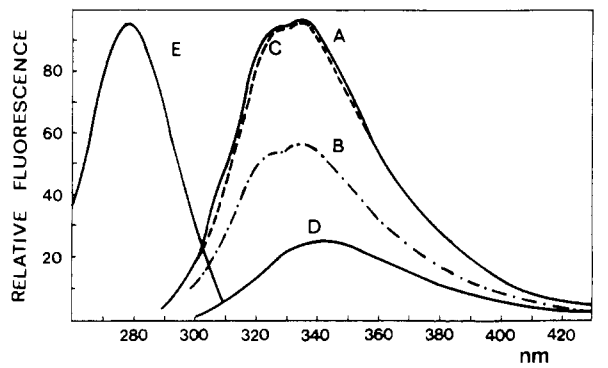

FIGURE 5: Fluorescence spectra of protein HC: (A) emission spectra for excitation at 284 nm; (B) emission spectrum for excitation at 295 nm; (C) same as (B) after normalization at longer wavelengths than 380 nm; (D) emission spectrum for excitation at 284 nm at pH 12.5; (E) excitation spectrum for emission at 327 or 337 nm. The protein preparations were dissolved in 0.1 M sodium phosphate buffer, pH 6.5, except for sample D, which was brought to the desired pH value by adding NaOH. Fluorescence values are plotted in arbitrary units.

calculated spectra, a positive contribution of about 3000 deg cm^2 $dmol^{-1}$ at 225 nm is obtained (Figure 1). Such a contribution could be due to the facts mentioned above.

Absorbance and Near-UV Circular Dichroism of Protein HC. Characterization of the tertiary structural level of protein HC has been carried out by absorbance, fluorescence, and CD measurements. The absorption spectrum of the protein is given in Figure 3. It shows a maximum at 278 nm as well as shoulders at 290, 270, and 260 nm. The A_{278}/A_{260} ratio is 1.6. The calculated extinction coefficient $E_{1cm,278}^{0.1\%}$ is 1.6, from use of amino acid analysis for determining protein concentration. A broad band can be observed in the visible region, which is related to the brown color of the protein. Many attempts have been performed to remove the color-associated material (Tejler & Grubb, 1976). Dialysis with guanidine hydrochloride and SDS (sodium dodecyl sulfate) is ineffective for this purpose. However, the band in the visible region is too broad and not well resolved to be assigned at only one chromophore.

The CD spectrum of protein HC in the 320–250-nm wavelength range is also given in Figure 3. The characteristic features of this spectrum are the negative bands centered at 282 and 289 nm and the positive bands at 255 and 310 nm. No significant anisotropy has been observed at longer wavelengths.

pH Titration of Human Protein HC. pH titration of protein HC has been followed by CD and absorbance measurements. The results are compiled in Figure 4. According to the ΔA_{295} values, the eight tyrosine residues of the molecule have been titrated. First-derivative analysis of the experimental points shows the existence of two inflection points at pH 10.3 and 12.0, and two groups of tyrosine residues are differentiated in protein HC, being composed of three and five residues, respectively. The second group with a pK value of 12.0 would correspond to buried residues. When the pH increases, the positive ellipticity band centered at 255 nm at neutral pH is blue-shifted to 250 nm. Taking this fact into account, if the ellipticity at 250 nm is considered as an index for the titration, two well-differentiated inflection points are located at pH 10.1 and 12.0, almost coinciding with those observed from the absorbance measurements. This curve does not fit a normal titration curve and must be the result of ionization of other groups, probably lysines, affecting protein conformation. If the titration is followed by the ellipticity at 205 nm as a secondary structure index, only one conformational transition is observed at pH 12.0. This is related to the denaturation of the protein, due to the significant increase of the θ_{205} ab-

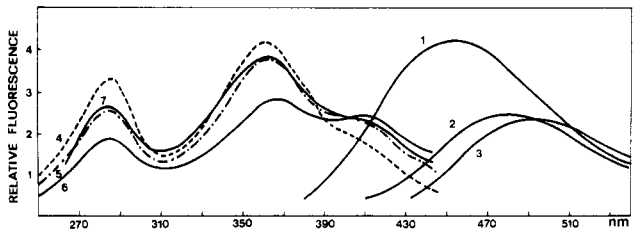

FIGURE 6: Fluorescence spectra of protein HC: (1–3) emission spectra for excitation at 360, 397, and 420 nm, respectively; (4) excitation spectrum for emission at 456 nm; (5) excitation spectrum for emission at 474 nm; (6) excitation spectrum for emission at 500 nm; (7) excitation spectrum for emission at 474 nm at pH 12.5. The protein preparations were dissolved in 0.1 M sodium phosphate buffer, pH 6.5, except for spectrum 7, which was brought to the desired pH value by adding NaOH. Fluorescence values are plotted in arbitrary units but are perfectly comparable with those employed in Figure 5.

solute value when the pH is increased. Thus, the group of tyrosine residues with pK = 12.0 is buried and becomes exposed when the protein is denatured at high pH values.

Fluorescence of Protein HC. The fluorescence spectra of protein HC are given in Figure 5. Upon excitation at 284 nm, two maxima at 327 and 337 nm, as well as a shoulder at 305 nm, are observed. This spectrum is interpreted in terms of two tryptophan residue populations located in microenvironments of different polarity. Upon excitation at 295 nm, the wavelength where the tyrosine residues exhibit less than 10% of their absorption properties (Teale, 1960), the fluorescence of Trp would be obtained. This spectrum is also given in Figure 5. When this spectrum is normalized at wavelengths longer than 380 nm, considering that the tyrosine fluorescence is negligible above 380 nm, the obtained spectrum is almost coincident with that observed for excitation at 284 nm. This result indicates a small contribution of tyrosine residues, located at 305 nm, to the fluorescence of protein HC (Figure 5).

When the excitation spectrum is recorded for emission at 327 and 337 nm, identical results are obtained (Figure 5). This fact could be interpreted in terms of an identical number of Trp residues at each of the two above-mentioned different populations. Thus, the four tryptophan residues on protein HC would be resolved in two groups of different microenvironment polarities, with two residues in each one.

Denaturation of the protein has been observed at pH 12.0 by following the titration through CD measurements at 205 nm. Effectively, when the emission spectrum of protein HC is recorded for excitation at 284 nm at pH values higher than 12.0, the observed quantum yield is sensibly decreased, and only one maximum at around 340 nm is obtained (Figure 5). This indicates that all the tryptophan residues are now in a homogeneous microenvironment of higher polarity than that in the native conformation. This red-shift in the fluorescence emission is clearly in agreement with the proposed denaturation process.

Fluorescence of the Chromophore Associated to Protein HC. The fluorescence of the chromophore associated to protein HC has been also studied. Excitation has been considered at three different wavelengths, 360, 397, and 420 nm. The emission spectra are given in Figure 6. The obtained results are completely different at each excitation wavelength. The excitation spectra are also recorded for different emission wavelengths (Figure 6). These results would correspond to the absorption properties of the chromophore associated to the polypeptide chain. Three different bands are observed at around 290, 360, and 410 nm. These spectra are not sensibly modified when the protein is denatured at pH higher than 12.0 (Figure 6), indicating that the chromophore is not dependent, with respect to its absorbance properties, on the protein conformation.

Discussion

Human complex-forming glycoprotein, heterogeneous in charge, has been purified from human urine and plasma, although it has been found in many biological fluids (Tejler & Grubb, 1976). The protein has been reported to be associated to lymphocyte cell surfaces (Tejler et al., 1976; Pearlstein et al., 1977), to possess immunoregulatory properties (Logdberg & Akerström, 1981), and to form complexes with IgA and albumin (Tejler & Grubb, 1976). However, its biological function remains largely unknown. Recently, we have reported the amino acid sequence of the protein (Lõpez et al., 1981), indicating that protein HC is related to, or identical with, the plasma protein α_1-microglobulin. However, the conformational properties of the molecule have not yet been studied. Considering the structure–function relationship of macromolecules, as well as the unknown function of protein HC, we have studied the conformational parameters of the protein.

The yellow-brown chromophore present in protein HC, which possibly is related to the presently unknown function of the protein, has been resolved in three different units according to their fluorescence properties. Also, this chromophore does not exhibit optical anisotropy as indicated by the absence of any CD signal in the visible region. Moreover, its spectroscopic properties are not modified at basic pH, even when the protein is denaturated. This indicates that the spectroscopic behavior of this chromophore is not dependent of the protein conformation because it is not modified upon protein denaturation. The spectroscopic characteristics of this chromophore with bands around 290, 360, and 410 nm do not correspond to any described chromophore. In addition, the three different fluorescent units, of which the spectrum of the yellow-brown chromophore can be resolved, could be responsible for the charge heterogeneity of the protein HC. On the other hand, other alternative explanations, referring to the assignation of the heterogeneity to the polypeptide chain or to the carbohydrate groups, have been discarded, since no variability of the amino acid sequence of protein HC was found (Lõpez et al., 1981) and no heterogeneity of the carbohydrate groups of α_1-microglobulin could be detected in a recent study (Ekström et al., 1981). This chromophore universally present on all preparations of protein HC seems not to be involved in covalent bonds to the polypeptide chain; at least during the sequence determination, this fact cannot be demonstrated. Moreover, this material adhered to most of the peptides of protein HC during their isolation. Consideration of this material as an artifact of the protein purification procedure is difficult because it is present in protein preparations from different sources and obtained by different purification procedures. Location of this chromophore on the protein is not easy because of the overlapping between the intrinsic fluorophore bands of the protein and those from that material. The chromophore cannot be related to the carbohydrate moiety of the molecule because carbohydrate-bound peptides from protein HC do not have the characteristic spectroscopic behavior of the chromophore (data not shown).

The secondary structure of the protein would be composed of 20% α-helix, 21% β-structure, and 59% aperiodic conformation. Considering the potential contributions of the nonpeptide moiety and the β-turns to the optical anisotropy of the molecule in the peptide bond region, both circular dichroism and theoretical predictive methods would give similar results

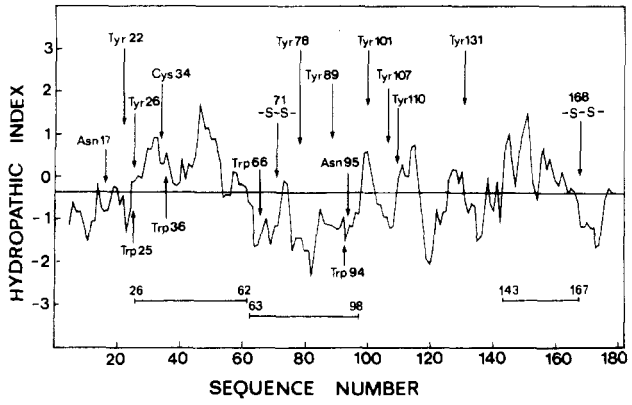

FIGURE 7: Hydropathic profile for protein HC (span setting of 9). Above the midpoint line corresponds to interior regions and below to external regions of the molecule, according to Kyte & Doolittle (1982).

concerning the secondary structure of the protein. Discussion of these results is difficult due to the absence of any homologous protein. A computer search with appropriate programs failed to reveal homologies in sequence between protein HC and any other known sequenced protein.

From attempts to corroborate the results obtained from the spectroscopies on protein HC, the hydropathic profile of the amino acid sequence has been obtained according to Kyte & Doolittle (1982). Variation of the hydropathic index vs. residue number is plotted in Figure 7 for a span setting of 9. This profile indicates the existence of three different well-defined domains, in order of exposure to the environment. A portion comprising of residues from positions 26–62 would be a buried domain. According to the prediction of secondary structure, this domain would be composed of two α-helix and one β-structure in the sequence α–β–α. A second buried domain would be extended from residue 143 to residue 167, which contains one α-region and one β-region. The third domain would be an exposed region of the molecule comprised of residues from position 63 to position 98. In this domain, there are only five residues in β-structure. Thus, the most exposed portion of the molecule contains the main part of the unordered conformation in protein HC. Other regions of the amino acid sequence cannot be easily organized in well-defined domains according to the hydropathic profile. From this profile, tyrosine residues located at positions 110, 101, and 26 would be located in buried regions. Tyrosine residues located at positions 78 and 89 will be on the outside of the molecule. Tyr residues 22, 107, and 131 are in the boundary position. This would be in agreement with the observation obtained from the titration experiment, where two different groups of tyrosine residues were observed, being composed of three and five residues corresponding to exposed and buried, respectively. Tryptophan residues located at positions 25 and 36 in the amino acid sequence would be buried according to this profile, whereas Trp residues at positions 66 and 94 would be in an exposed region. These observations would be in agreement with the results obtained from the fluorescence spectroscopy study, which indicates the existence of two tryptophan residue populations, with different polarity microenvironments, each of them being composed of two Trp residues. The carbohydrate component is bound to Asn residues in positions 17 and 95, which are in exposed regions according to the hydropathic profile. The only disulfide bridge in the molecule, formed between cysteine residues at positions 71 and 168, bound two exposed regions of the molecule. However, the cysteine residue at position 34, which has been proposed (Méndez et al., 1982) to be involved in disulfide bridges with cysteine and other components of unknown structure, is located in a buried region of the molecule. Taking into account that this cysteine residue has been suggested to be involved in the function of protein HC, mediating the formation of complexes with other plasma proteins such as IgA and albumin (López et al., 1981), an active site of the molecule related to this residue would be located in an internal region of the protein.

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