Solubility of Heme in Heme-Iron Enriched Bovine Hemoglobin Hydrolysates

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A heme-iron enriched peptidic hydrolysate was prepared from bovine hemoglobin, at pilot plant scale, by peptic hydrolysis followed by ultrafiltration. Such preparations are attractive for iron deficiency therapy and have been reported in the literature in the context of utilization of blood in the food industry. The peptidic fraction of this hydrolysate was able to solubilize heme in higher proportion than hemoglobin even at acidic pH where heme is totally insoluble. One peptide, having a similar ability to solubilize heme in the same range of pH, was isolated from this fraction and taken as a model to investigate the mechanisms involved in heme solubilization. Heme seemed to be mainly solubilized through hydrophobic interactions with the peptide, whereas ligancies or electrostatic interaction could not be demonstrated. The stoichiometry of heme−peptide adducts depends on pH with a 2:2 association at pH 2 between heme as a dimer and two peptides and with a 2:1 association at pH 7.5 between one dimer and one single peptide. However, the existence of higher molecular weight aggregates cannot be excluded in the whole hemoglobin hydrolysate. Despite the good solubility of heme and the high heme/protein ratio, such heme enriched peptidic hydrolysates could have a weak bioavailability since heme polymerization is known to decrease heme-iron intestinal absorption. Further studies will be necessary to reduce heme polymerization during enzymatic hydrolysis of bovine hemoglobin.

Keywords: *Globin hydrolysate; heme-iron; hydrophobic interaction; iron deficiency*

INTRODUCTION

Iron deficiency is known to be one of the most common nutritional disease, and several millions of people are classified as iron deficient. The highest prevalence is in developing countries where the most frequent causes are poor iron bioavailability from the predominantly cereal diets and blood loss due to parasitic infestations. In the industrialized countries, iron anemia occurs among people having large iron requirements due to growth as infants, fetal demands as pregnant women, or iron loss as menstruating women and adolescent girls (Carpenter and Mahoney, 1992). Ferrous sulfate is widely used to prevent and treat anemia. However, common side effects of this therapy include nausea, epigastric discomfort, abdominal cramps, constipation, and diarrhea. For these reasons, new iron-based preparations are needed that are both well absorbed and tolerated.

Another source of iron is heme-iron, which is found in the diet as myoglobin or hemoglobin. Hemoglobin iron is assimilated better than iron salts (Turnbull et al., 1962; Callender et al., 1957), and many studies have shown that absorption of heme-iron is not dependent upon dietary composition (Conrad et al., 1967) and produces little side effects (Frykman et al., 1994). Peptides produced by proteolytic digestion of hemoglobin or other dietary proteins would maintain heme in a soluble form so that it would remain available for absorption (Uzel and Conrad, 1998).

In animal blood, hemoglobin accounts for more than half of the blood proteins and 90% of the cell fraction proteins. Most of the slaughterhouse hemoglobin remains an agricultural waste product and could be, in the future, an inexpensive source of heme-iron for developed and developing countries. Numerous researchers have attempted to prepare hemoglobin heme-iron. Whole hemoglobin was successfully used in cookies or as pills to alleviate childhood iron deficiency (Walter et al., 1994; Mitsubishi, 1987). The low proportion of iron in hemoglobin limits its use in treatment of iron deficiency. Many patents pertain to the development of processes to produce pure heme from hemoglobin based on treatments with acids (Autio et al., 1983), organic solvents (Luijerink, 1983; Lindroos, 1981), or proteases (Mitsubishi Chemical Industry Co., 1983). Insoluble heme preparations were obtained and often added to chocolate for treating anemia. However, the bioavailability of such products seems to be largely affected by the high polymerization of the heme in the gastrointestinal tract (Carpenter and Mahoney, 1992; Reinzenstein, 1990), and their insolubility in water limits their applications.

Another approach to overcome these problems is to prepare heme-iron peptidic hydrolysates from hemoglobin by enzymatic hydrolysis followed by ultrafiltration to accumulate heme-iron enriched fractions (Erikson, 1981; Lebrun et al., 1998). These heme-enriched hydrolysates have a higher iron content than hemoglobin, and their iron complexed with peptides. Limited data, however, are available regarding the water solubility of these heme−peptide complexes. There is the question

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of whether the origin of the heme−peptide interactions can be manipulated to improve their nutritional applications.

This paper describes the preparation of a soluble peptic hydrolysate enriched in heme-iron from bovine hemoglobin. The hydrolysates were studied, regarding the mechanisms involved in the heme solubilization and the nature of the interactions between heme and peptides. A peptide was isolated by HPLC and identified by amino acid analysis and mass spectrometry, and heme−peptide interactions were characterized at acidic and slightly basic pH. The heme was mainly linked with peptides through hydrophobic interactions. This study has some interest in the context of the utilization of bovine hemoglobin, an agricultural byproduct, for the prevention of anemia.

MATERIALS AND METHODS

First, the method to prepare the heme-iron enriched bovine hemoglobin hydrolysate at the pilot plant scale is described. A decolorization method of the hemoglobin hydrolysate was performed to compare solubility of heme in the hydrolysate with that of the peptidic fraction. Reversed-phase HPLC, mass spectrometry, and amino acid analysis were used to isolate, identify, and characterize a peptide of this fraction. A method to evaluate the ability of the peptide to solubilize hemin is described. Interaction of the peptide and hemin were studied by UV−vis difference spectrometry and flurescence spectrometry. A model based on a simple equilibrium between the peptide and hemin as a monomer or as a dimer was used to estimate dissociation constants of the complexes.

Preparation of the Heme-Iron Enriched Hemoglobin Hydrolysate. Centrifuged bovine red cells were provided by Veos n.v. (Zwevezele, Belgium). Hemoglobin hydrolysate was prepared at pilot plant scale as previously reported (Lebrun et al., 1998). Hemoglobin solution was prepared by hemolysing 1 vol of erythrocytes with 3 vol of 10 mM HCl. Hemoglobin concentration was determined according to the cyanmethemoglobin method (Crosby et al., 1954). Eighty liters of hemolysate at 50 g/L was brought to 40 °C in a thermostated reactor (Inceltech LH.SGI, Toulouse, France), and pH was adjusted to 4 with 4 M HCl. The enzymic digestion was started by the addition of porcine pepsin (Sigma Chemical Co., St Louis, MO) at an enzyme/protein ratio of 1.4% (w/w). The pH was maintained at 4 by the addition of 4 M HCl using a pH stat (Inceltech LH.SGI). After 24 h, the hemoglobin hydrolysate was clarified by centrifugation, and pepsin was inactived by raising the pH to 8 with concentrated NaOH. The degree of hydrolysis determined by the trinitrobenzene sulfonate method (Addler-Nielsen, 1979) was 11%. Seventy liters of clarified hemoglobin hydrolysate was reduced to 35 L by ultrafiltration with a transmembrane differential pressure of 10^5 Pa. The retentate was then diafiltered with 3 vol of water. The heme/peptide ratio in the heme-iron enriched retentate was up to 7% (w/w), i.e., more than twice as high as in hemoglobin. Molecular masses of the peptides determined by urea−SDS−polyacrylamide gel electrophoresis according to the method of Anderson et al. (1983) were about 4−5 kDa. The enriched heme-iron hydrolysate was collected and spray-dried.

Decolorization of the Peptidic Hemoglobin Hydrolysate. The resolubilized heme-iron enriched hydrolysate powder was decolorized with acetone at acidic pH (Antonini and Brunori, 1971). The hemoglobin hydrolysate was treated at 20 °C with acetone containing 2−3 mL of 2 N HCl/L. Twenty milliliters of the 3−5% (w/v) heme-iron peptidic hydrolysate was gradualy added with vigorous stirring to about 250 mL of the acetone solution. Heme was solubilized in acetone, and the precipitated peptidic fraction was collected by centrifugation and freeze-dried as a peptidic powder.

Study of the Solubility of Heme and Peptide. The enriched heme-iron hydrolysate (peptide, 50 mg/mL; heme, 0.35 mg/mL), the decolorized peptidic fraction (50 mg/mL), or pure hemin (0.35 mg/mL) was solubilized in 4 mL of 50mM NaOH. The pH of the solution was adjusted to the appropriate pH (2−12) with diluted HCl. The solutions were shaken and centrifuged at 5000*g* for 20 min. The volume of the supernatant was readjusted to 4 mL with water. Aliquots of each sample were assayed for heme content according to the pyridine hemochromogen method (Paul et al., 1953).

Reversed-Phase High-Performance Liquid Chromatography. Purification of the peptide from the decolorized peptidic hemoglobin hydrolysate was performed by reversed-phase HPLC on a semipreparative C18 column (250 mm × 10 mm.; particle size 5 μm) (Vydac, Hesperia, CA). Peptidic samples were dissolved in eluent A (5 mg/mL), filtered through a 0.2 μm pore-size filter, and injected into the column. Peptides were eluted at 3.5 mL/min with a gradient of 0.1% (w/v) trifluoroacetic acid in water as eluent A and acetonitrile with 0.1% TFA as eluent B. A linear gradient from 19% to 50% B was carried out in 124 min. Peptides were monitored at 226 nm. The peptidic fraction was collected and freeze-dried.

Mass Spectrometry of the Peptide. Dried peptidic sample was dissolved at a concentration of 20 pmol/μL in a solution containing 20% (v/v) acetonitrile and 0.1% (v/v) formic acid in water and a 10 μL sample was injected into a carrier solvent of the same composition. Ion spray mass spectra were recorded with a single-quadrupole mass spectrometer (Perkin-Elmer, Thornhill, Canada) equipped with an ion spray (nebulizer-assisted electrospray) source (Sciex, Toronto, Canada). The solutions were continuously infused with a medical infusion pump (model 11, Harvard Apparatus, South Natick, MA) at a flow rate of 5 μL/min. Polypropylene glycol was used to calibrate the quadrupole. Ion spray mass spectra were acquired at unit resolution by scanning from m/z 400 to m/z 1800 with a step size of 0.1 Da and a dwell time of 2 ms. The potential of the spray needle was held at 4.5 keV, and the spectra were recorded at an orifice voltage of 60 V. Calculation of the molecular masses of the samples was performed on a Mac Bio Spec computer program.

Amino Acid Analysis. Amino acid composition of the peptide was determined using a Picotag Work Station (Waters, Milford, MA). Peptide (5 μg) hydrolysis was carried out with constant boiling HCl (containing 1% phenol) for 24 h at 110 °C. Precolumn derivatization of amino acids with phenylisocyanate and HPLC separation of derivatized amino acids on a RP-Picotag column (150 mm × 3.9 mm) (Waters) were performed according to the method described by Bidlingmeyer et al. (1984). Absorbance was measured at 254 nm using a Waters model 455 detector.

Solubilization of Hemin by the Peptide at Acidic pH. The peptide was dissolved in 2 mL of a mixture containing 75% (v/v) acetone and 25% (v/v) 50 mM HCl−KCl pH 2 buffer. Two milliliters of this mixture containing hemin (Sigma, St. Louis, MO) was added to the 2 mL peptide solution. Final concentration of the peptide was 4 μM. Final concentrations of hemin were varied from 0 μM up to 12 μM. Acetone was removed by vacuum evaporation. The insoluble residues were discarded by centrifugation (5000*g* for 15 min). To determine the amounts of soluble hemin−petide complex; the volume of the resultant solution was adjusted to 4 mL with acetone, and its absorbance was measured at 385 nm. A calibration curve was determined with free hemin in the same acetone−buffer mixture.

UV−Visible Difference Spectrometry. Hemin was dissolved in 0.1 M sodium hydroxide at a concentration of 2 mM. The hemin solution was further diluted to 9.8 μM with 50 mM Tris-HC1 buffer, pH 7.5. Difference spectra were recorded on a lambda 5 spectrophotometer (Perkin-Elmer). Five microliter increments of a peptide solution (1.95 mM in the Tris-HCl buffer) were added to the 3 mL sample cell (final peptide concentrations were varied from 3.2 to 44.8 μM), while equivalent volumes of buffer were added to the reference cell. After each addition, the difference spectrum was recorded. Absolute spectra were recorded at the begining and at the end of each experiment.

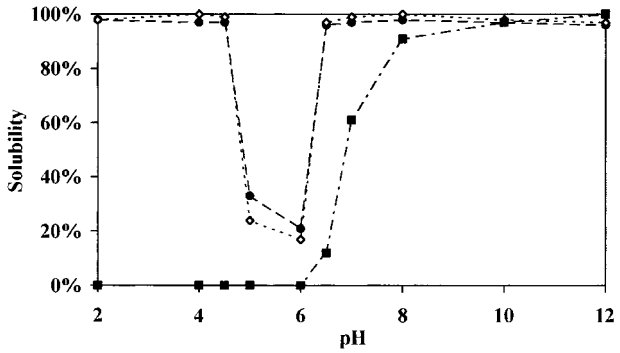

Figure 1. Solubility of heme and peptides as a function of pH. (◊) Heme of the enriched heme-iron hydrolysate; (■) pure hemin; (●) peptidic fraction of the hydrolysate after removing heme. Solubility is expressed in percentage of heme or peptides solubilized at a given pH in comparison with the initial amount solubilized in 50 mM NaOH.

Evaluation of the Dissociation Constants of Hemin−Peptide Complexes. A simple equilibrium may be represented by

$$\mathbf{H} + \mathbf{P} \rightleftarrows \mathbf{HP} \qquad \text{with } K = \mathbf{H}\cdot\mathbf{P}/\mathbf{HP} \tag{1}$$

where **H**, **P**, **HP**, and K are respectively the concentrations at the equilibrium of hemin as a dimer (or heminCN as a monomer), peptide, hemin−peptide complex, and its dissociation constant. Considering that $\mathbf{H_0}$ and $\mathbf{P_0}$ are respectively the total concentration of heme (as a dimer or as a monomer) and peptide in the solution, the dissociation constant can be written from eq 1 as $K = (\mathbf{H_0} - \mathbf{HP})(\mathbf{P_0} - \mathbf{HP})/\mathbf{HP}$. Since in our experimental conditions $\mathbf{P_0}$ is not much larger than $\mathbf{H_0}$, the Eadie Scatchard representation is not appropriate, and we must calculate the concentration of hemin−peptide **HP** complex as a function of $\mathbf{H_0}$ and $\mathbf{P_0}$:

$$\mathbf{HP} = [(K + \mathbf{H_0} + \mathbf{P_0}) - ((K + \mathbf{H_0} + \mathbf{P_0})^2 - 4\mathbf{H_0}\mathbf{P_0})^{1/2}]/2 \tag{2}$$

By measuring the changes in absorbance, ΔA, at 417 nm of the difference spectra corresponding to variable concentrations of the peptide (from 3.2 to 44.8 μM) for a constant hemin concentration (9.8 μM as a monomer or 4.9 μM as a dimer), $\Delta A/\Delta A_{max}$ can be determined, where ΔA_{max} is the maximal change of absorbance corresponding to complete binding of hemin. Then $\Delta A/\Delta A_{max} = \mathbf{HP}/\mathbf{H_0}$ and from eq 2, we can write the following: $\Delta A = \Delta A_{max}\,[[(K + \mathbf{H_0} + \mathbf{P_0}) - ((K + \mathbf{H_0} + \mathbf{P_0})^2 - 4\,\mathbf{H_0}\mathbf{P_0})^{1/2}]/2\mathbf{H_0}]$. As it is difficult to estimate ΔA_{max}, a quasi-Newton fitting algorithm with two unknown constants (K and ΔA_{max}) was used to fit our experimental results (ΔA, $\mathbf{P_0}$, and $\mathbf{H_0}$).

Fluorescence Spectrophotometry. Fluorescence spectra of the tryptophan containing peptide were measured at a constant 5 μM peptide concentration, and hemin concentration was varied from 0 to 5 μM. Excitation was performed at 280 nm. The fluorescence emission spectra of the peptide were monitored between 300 and 400 nm on a LS 50B spectrophotofluorimeter (Perkin-Elmer).

RESULTS

Heme Solubility in the Heme Enriched Hemoglobin Hydrolysate. Comparison of the solubility of the heme alone as hemin and of the heme as a hemoglobin hydrolysate was achieved between pH 2 and pH 12. As described in Figure 1, pure hemin was only soluble above pH 8, whereas in the same conditions heme in the form of hemoglobin hydrolysate remained soluble in a large range of pH (2−12), except around pH 5.5, which corresponds to the precipitation of the peptides of the decolorized hemoglobin hydrolysate. These precipitations probably correspond to the mean isoelectric pH of the peptides. Several authors (Léonil et al., 1994; Erickson, 1981) have already observed these precipitations and explained them by a decrease of the charge of the peptides and the formation of hydrophobic interactions. These results clearly prove that, inside the hemoglobin hydrolysate, heme was solubilized through interactions with the peptidic fraction, especially at acidic pH where hemin alone is completely water-insoluble. To understand better the mechanism of the heme solubilization and the origin of the heme−peptide interactions in this heterogeneous peptidic hydrolysate, a peptide able to solubilize heme in the same pH range as the whole heme enriched hydrolysate was isolated to study its interaction with heme.

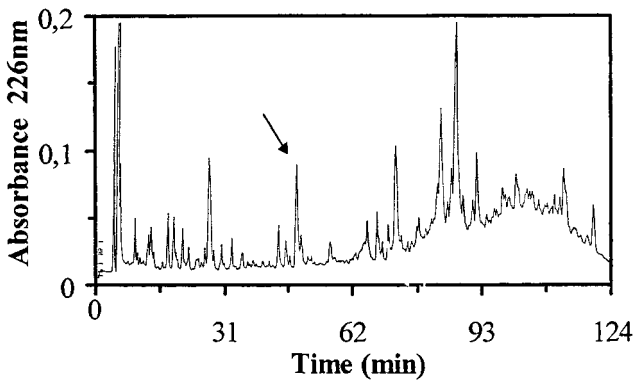

Figure 2. Semipreparative RP-HPLC separation of the selected peptide. The decolorized heme-iron hemoglobin hydrolysate was chromatographied on a Vydack (250 mm × 10 mm) C18 column. Solvent A, TFA 0.1% in H_2O. Solvent B, acetonitrile with 0.1% TFA. Gradient, 19−50% B in 124 min. Flow rate, 3.5 mL/min. The arrow indicates the selected peptide.

Isolation of a Peptide from the Heme Enriched Hemoglobin Hydrolysate. In the course of ultrafiltration of the peptic hemoglobin hydrolysate, numerous peptides passed through the membrane. Reversed-phase chromatography showed that this peptidic fraction is more hydrophilic than that of the retentate; moreover, it did not solubilize heme (Lebrun et al., 1998). A semipreparative reversed-phase chromatography of the heme enriched fraction of the retentate was performed on a C18 column (Figure 2). At small retention times, the chromatogram exhibited a good resolution of the peptides. At high retention times, the chromatogram exhibited a poor resolution in the most hydrophobic range, despite a real effort in chromatography optimization. A size exclusion chromatography study showed that an important part of the peptides was excluded from a G50 Sephadex column, proving the formation of peptide−peptide associations that were disrupted only with strong dissociating agents such as 5 M guanidine chloride (Lebrun et al., 1998). Such associations have already been reported by Liu et al. (1996) with high molecular weight peptides of globin hydrolysates prepared from porcine hemoglobin. For the following study and to avoid further peptide−peptide associations that could disturb the study of hemin−peptide interactions, we selected a peptide in the well-resolved hydrophobic fraction, among most the abundant peptides to make its purification at the preparative scale easier. The chosen peptide was eluted at 30.8% acetonitrile, collected, freeze-dried, and analyzed by electrospray ionization mass spectroscopy.

The transformed ion spray mass spectrum of the peptide (Figure 3) showed a large peak at 3176 Da. The

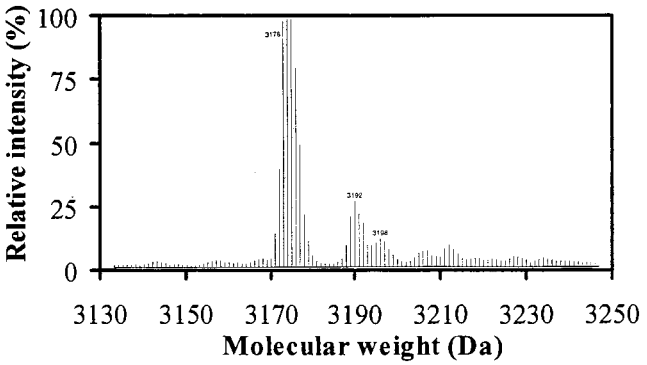

Figure 3. Transformed ion spray mass spectrum of the selected peptide. The peptide was dissolved at a concentration of 20 pmol/μL in a solution containing 20% (v/v) acetonitrile and 0.1% (v/v) formic acid in water, 10 μL was injected into a carrier solvent of the same composition.

Table 1. Amino Acid Composition and Deduced Molecular Mass of the Selected Peptide from Pepsic Bovine Hemoglobin Hydrolysate[a]

amino acid composition of the peptide												
Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Leu	Phe	Lys	Arg	Trp
7	6.7	14	13.7	15.7	15.8	11	2.8	10	4.1	8.5	5.8	nd

[a] Amino acid composition is expressed as g of amino acid/100 g of total amino acids. Deduced molecular weight, 3179 Da.

presence of the smaller peaks were consistent with probable oxidation for the 3192 Da peak and with the formation of sodium salts corresponding to the peak at 3198 Da.

In bovine hemoglobin structure, the mass of the selected peptide (3176 Da $\pm$ 1) may correspond to three amino acid sequences: α (Thr_{67}-Pro_{95}), β(Met_1-Leu_{30}), and finally β(Met_{74}-Asn_{101}). Identification of the peptide was acquired from the amino acid analysis. The molecular mass (3179 Da) deduced from amino acid composition (Table 1) was close to that determined by mass spectroscopy (3176 Da), demonstrating the purity of the isolated peptide and indicating the following sequence, which represents fragment 1−30 of the primary structure of bovine hemoglobin β chain:

^{1}Met-Leu-Thr-Ala-Glu-Glu-Lys-Ala-Ala-^{10}Val-Thr-Ala-Phe-Trp-Gly-

Lys-Val-Lys-Val-^{20}Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg-^{30}Leu

In the bovine hemoglobin conformation, this sequence consists of two α-helical regions, helix I [Thr_3-His_{18}] and helix II [Asp_{20}-Leu_{30}], which are connected by an hinge amino acid, Val_{19}. Schematic ribbon drawing of the peptide is shown in Figure 4. The average hydrophobicity $H\Phi_{ave}$ of the peptide (905 cal/mol) calculated according to the method of Bigelow (1967) was similar to that calculated for the whole hydrolysate (939 cal/mol).

Solubilization of Heme by the Pure Peptide. The ability of the peptide to solubilize heme in aqueous solution was studied with hemin at acidic pH for which free hemin is not soluble. Hemin and peptide were solubilized in a water−acetone solvent (25/75, v/v) at pH2. Acetone was removed by vacuum evaporation, and hemin remaining in solution was determined. Hemin solubilizations were carried out, for a constant peptide concentration (4 μM) in the water−acetone solvent, by increasing hemin concentrations up to 12 μM. As seen in Figure 5, the concentration of water-soluble hemin increased as a function of the initial concentration of

Figure 4. Schematic ribbon drawing of the selected peptide. This figure is drawn by the RAS MOL program from the spatial conformation of bovine hemoglobin β chain.

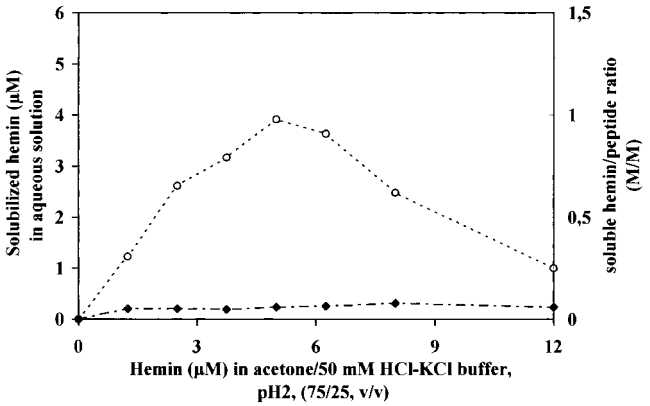

Figure 5. Solubilization of hemin by the peptide at pH 2 as a function of hemin concentration. Hemin concentration was varied from 0 to 12 μM, for a constant 4 μM peptide concentration in the acetone/HCl buffer mixture (75/25, v/v). (○) Hemin solubilization by the peptide; (♦) blank (hemin without peptide).

hemin in the water−acetone solvent. The maximal solubilization of hemin was obtained for hemin−peptide ratio near 1:1 (mol/mol), whereas free hemin at the same conditions was totally insoluble.

For a hemin concentration higher than 5 μM in the water−acetone solvent, the ability of the peptide to solubilize hemin dramatically decreased, and hemin was pratically water-insoluble from a 3:1 hemin−peptide ratio. These results clearly show that this pure peptide alone, isolated from the heme enriched peptidic hydrolysate, can solubilize heme very efficiently in aqueous solutions with an apparent stoichiometry of 1:1. At this proportion the heme−peptide ratio expressed as weight is 20%, whereas it is only 7% in the heme enriched peptidic hydrolysate. So, in a first approach, the

interactions of this peptide with heme could be representative of the heme−peptide interactions occurring with the peptides that carry heme in solution in the hydrolysate. We have undertaken to study the mechanism of heme solubilization of this peptide in aqueous solution.

Study of Heme−Peptide Interactions. The study of the hemin−peptide interactions by UV−visible spectrometry in aqueous solution at acidic pH is not possible because hemin is insoluble, and the formation equilibrium of the heme−peptide complex cannot be studied. So, this study was carried out at pH 7.5 for which heme and peptide are both soluble. The UV−vis spectrum of hemin at pH 7.5 in 50 mM Tris-HCl exhibited a broad Soret band centered at 363 nm (Figure 6). This band was attributed to intermolecular heme−heme associations because porphyrins are known to polymerize in aqueous solutions (Margalit and Rotenberg, 1984). In a mixture of ethylene glycol/50 mM Tris-HCl, pH 7.5 (50% v/v), the Soret band shifted to 417 nm and narrowed, showing the dissociation of heme polymers to monomers. By addition of the peptide to hemin solution at a molar heme−peptide ratio of 1:4 in 50 mM Tris-HCl buffer, pH 7.5, the Soret band shifted from 363 to 417 nm with a shoulder remaining at 363 nm. This shift could be due to hemin−peptide interactions. The same spectrum was observed with the whole heme enriched hemoglobin hydrolysate (data not shown).

The peptide contains a tryptophan residue (at the 14th position) that can be used as a fluorescent marker of the hemin−peptide interaction. To confirm these interactions, the wavelength of the fluorescence emission maximum of the peptide (5 μM) in the absence and in the presence of increasing concentrations of hemin (1−5 μM) in 50 mM Tris-HCl, pH 7.5, buffer was measured (Figure 7). The wavelength of the fluorescence emission maximum of the peptide alone was 356.5 nm. In the presence of hemin, a slight blue shift about 5 nm can be observed in the emission maximum at a hemin−peptide ratio of 1:1 and a decrease of the emission intensity. This blue shift typically occurs when tryptophan enters a more hydrophobic environment (Cajal et al., 1996). The decrease of the emission intensity can be probably assigned to the reabsorption of the light by hemin added to the medium. Thus, it is difficult to study in detail the heme−peptide interaction by fluorescence spectroscopy.

To characterize these interactions better, difference absorption spectrophotometry was used. Difference spectra for hemin−peptide associations at pH 7.5 vs heme alone as a function of peptide concentrations are shown in Figure 8. The difference spectra clearly exhibited defined isobestic points with a trough at 363 nm and a peak at 417 nm. This result shows the existence of an association equilibrium between hemin and peptide in aqueous solution at pH 7.5. To test the role of the iron in the heme−peptide interaction, difference spectra were performed by addition of the peptide to heminCN (Figure 9). The difference spectra, as in the case of hemin in absence of ligand, showed defined isobestic points, which proved that an association equilibrium between hemin and peptide was involved even when the iron binding sites were liganded. On the other hand, no significant difference spectrum was observed (Figure 10) when the peptide was added to a hemin solution in a mixture of ethylene glycol and 50 mM Tris-HCl buffer, pH 7.5 (50% v/v).

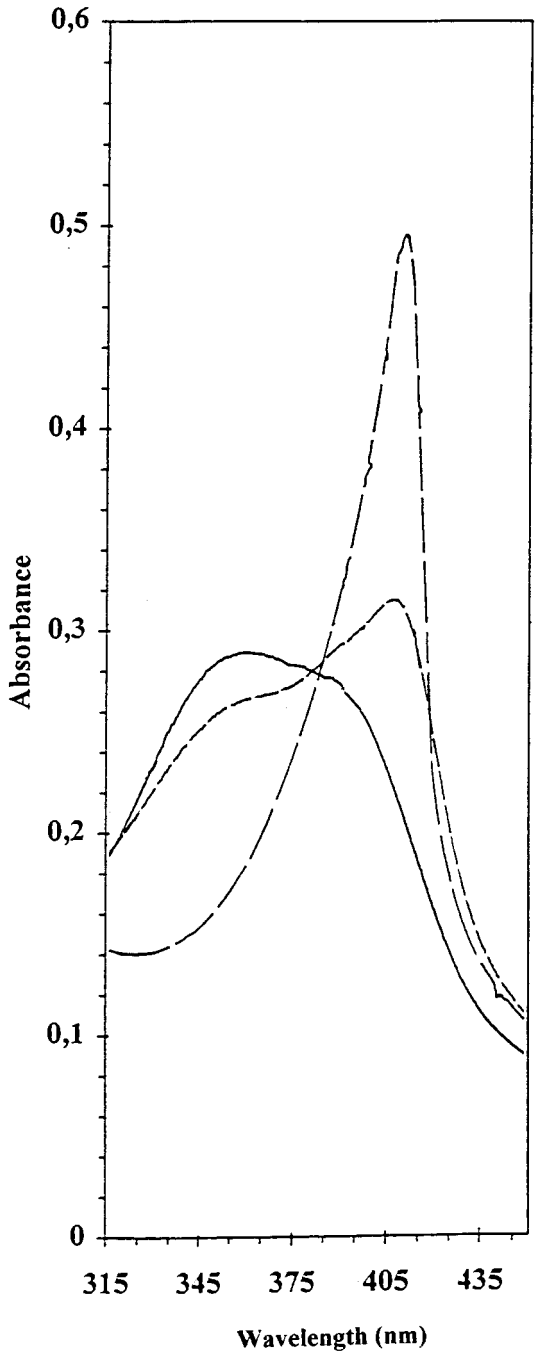

Figure 6. UV−vis spectra of hemin. (—) Hemin in 50 mM Tris-HCl, pH 7.5; (---) hemin−peptide complex in 50 mM Tris-HCl, pH 7.5; (— —) hemin in a mixture containing 50% (v/v) 50 mM Tris-HCl buffer, pH 7.5, and 50% (v/v) ethylene glycol.

DISCUSSION

Heme solubility as a function of pH is directly related to the solubility of the peptides as shown by the solubility curve of the peptidic fraction after removing of heme (Figure 1) and the very weak solubility of both heme and peptides observed at the isoelectric pH. Moreover, the peptide of 30 amino acids isolated from the hemoglobin hydrolysate exhibited a high ability to solubilize hemin at acidic pH with a hemin peptide ratio of 1:1 (mol/mol). Study of the interaction of this peptide with hemin would allow for a better understanding of how heme is solubilized in heme enriched hemoglobin hydrolysate. Further developments of hemoglobin pro-

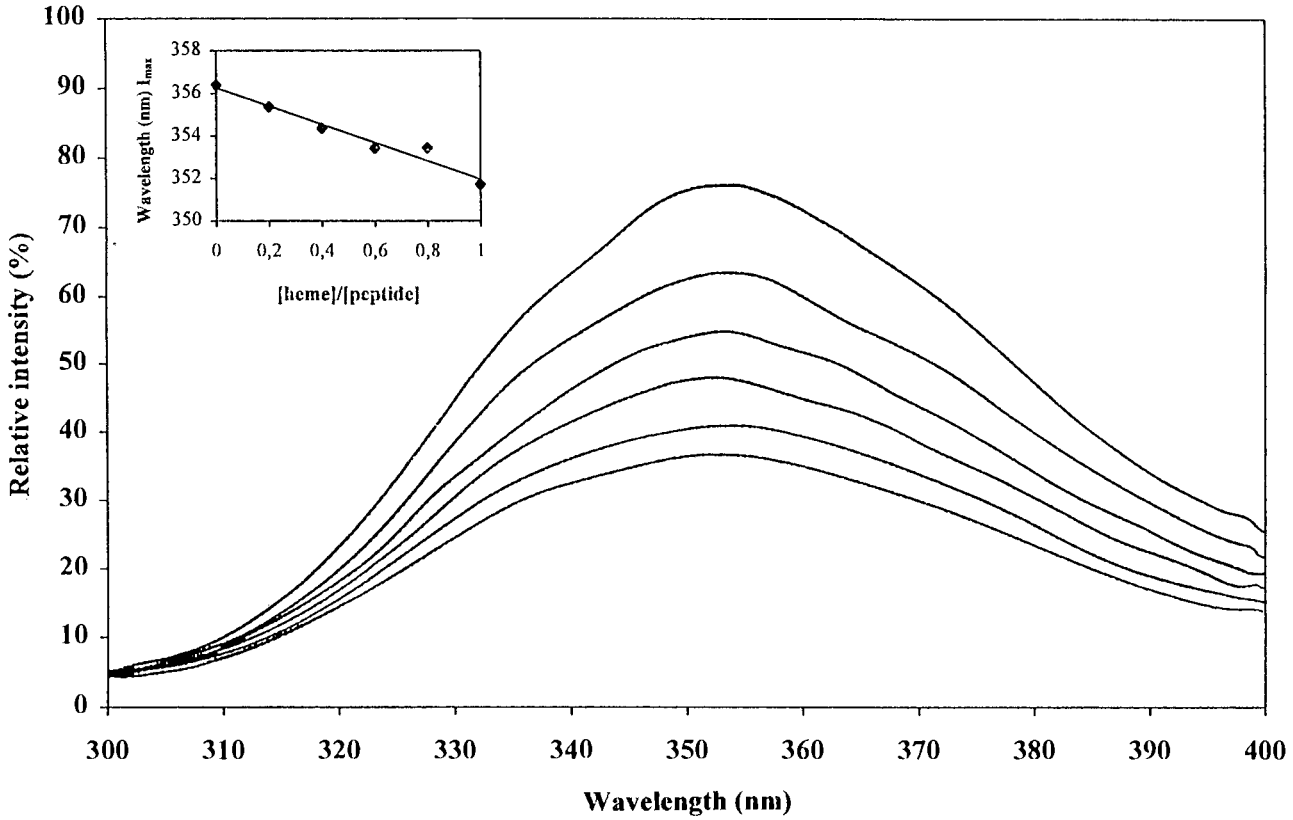

Figure 7. Displacement of the fluorescence emission maximum of the tryptophan containing peptide during hemin-peptide association. Conditions: 5 μM peptide; 0−5 μM hemin in 50 mM Tris-HCl buffer, pH 7.5, 22 °C; excitation wavelength: 280 nm. Insert: Shift of the emission maximum as a function of the heme/peptide ratio ($r^2 = 0.996$).

duced by various processes and hydrolysates for prevention or treatment of iron deficiency will depend on an improvement of the understanding of the peptide−heme interactions that are in relation to the bioavailability of heme-iron.

The question of the stoichiometry of the heme−peptide complex can be raised. Ferriheme is known to form aggregates in aqueous solution, even for weak concentrations (Brown and Shillock, 1976; Leclerc et al., 1993). An equilibrium dissociation constant of 5×10^{-9} M was reported for the dimerization of heme at pH 7.7 by Bakes et al. (1986). In our conditions, at pH 7.5 and for a hemin concentration of 9.8 μM, with the same dissociation constant, more than 98% of hemin would be in the form of dimers (i.e., about 4.9 μM as a dimer). The UV−visible spectrum of hemin at pH 7.5 (Figure 6) confirms the existence of heme−heme associations by exhibiting a broad Soret band centered near 362 nm. In the presence of ethylene glycol, a breaker of hydrophobic interactions, the spectrum was consistent with that of monomeric heme with a tight Soret band and an absorption maximum at 417 nm. In the presence of peptide at a molar peptide−heme ratio of 4:1 (mol/mol), the Soret maximum shifted to 417 nm, but the Soret band remained very broad with a shoulder at 362 nm (Figure 6). This shift could be assigned to a partial dissociation of heme aggregates to monomers due to the peptide; but even for a large excess of peptide, the aspect of the Soret band remained unchanged. So we can assume that the changes observed in the hemin spectrum by addition of peptide could be explained by a peptide−dimer interaction.

The difference spectra observed in the course of the interaction of the peptide with hemin in aqeous solution at pH 7.5 could be explained by a simple equilibrium between the peptide and hemin as a dimer. A good fitting of the data ($r^2 > 0.99$) was obtained with this assumption, and a dissociation constant of 1.5×10^{-5} M was estimated (Figure 8, insert B). For the interaction between heminCN, which is in the form of a monomer, and Masporan, a 14 amino acid peptidic toxin, a very close value was reported by Leclerc et al. (1993) with a dissociation constant of 10^{-5} M.

We can summarize the formation of the peptide−hemin complex at pH 7.5 as

$$2 \text{ hemins} \rightleftarrows \text{dimer} \qquad K_D = 5 \times 10^{-9} \text{ M}$$

$$\text{peptide} + \text{dimer} \rightleftarrows \text{peptide−dimer} \qquad K_D = 1.5 \times 10^{-5} \text{ M}$$

Consequently, in aqueous solution at pH 7.5, the peptide−hemin ratio in the complex would be 1:2 (mol/mol). The formation of hemin−peptide adducts at pH 2 by the acetone method led to complete solubilization of hemin up to only a peptide−hemin ratio of 1:1 (mol/mol) in the water−acetone solvent; for higher ratios, hemin was not completely solubilized, and a part of hemin precipitated with the peptide (data not shown). Moreover, the UV−visible spectrum of peptide-hemin adducts at pH 2 exhibited a broad Soret band (result not shown) showing that hemin was in the form of dimers. Consequently, at acidic pH, to explain these results, we must assume the formation of peptide−heme associations with the 2:2 stoichiometry (mol/mol). The peptide−dimer association (peptide−hemin ratio of 1:2, mol/mol) would not be formed or would not be water soluble in acidic medium

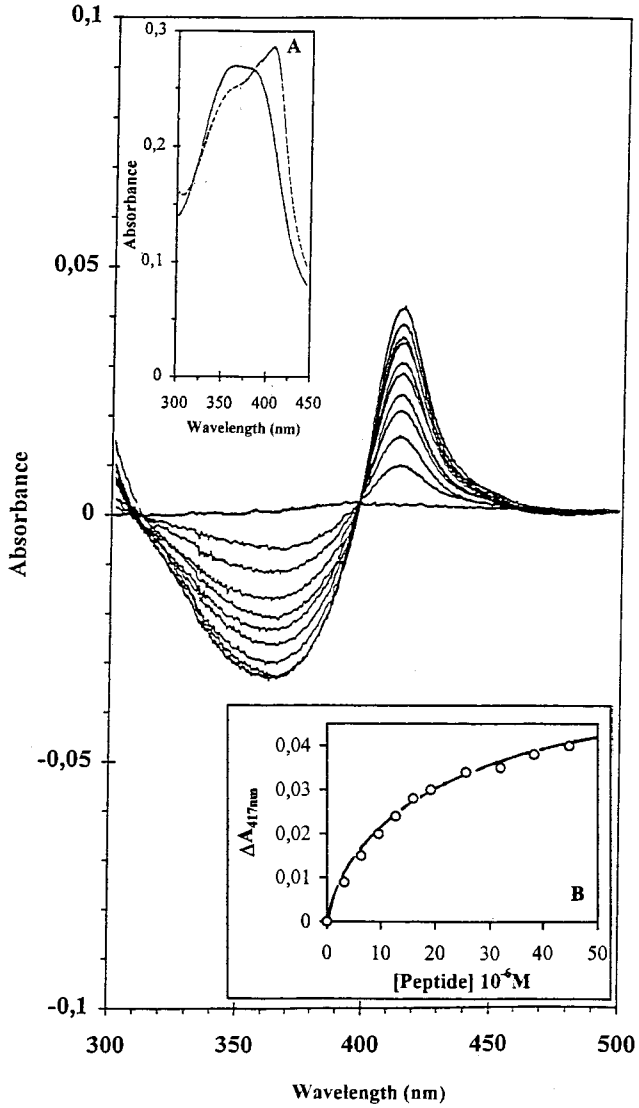

Figure 8. Difference spectra resulting from peptide−hemin interactions. Peptide was diluted to a stock concentration of 1.95 mM in 50 mM Tris-HCl buffer, pH 7.5. 5 μL increments were added to the 3 mL sample cuvette containing the hemin solution (9.8 μM in 50 mM Tris-HCl, pH 7.5), while equivalent volumes of the same buffer were added to the reference. The final peptide concentration ranged from 3.2 to 44.8 μM. Insert (A): Absolute spectra of the hemin solution before addition of peptide (—) and after the last addition of peptide (---). Insert (B): Optical titration of the peptide at 417 nm.

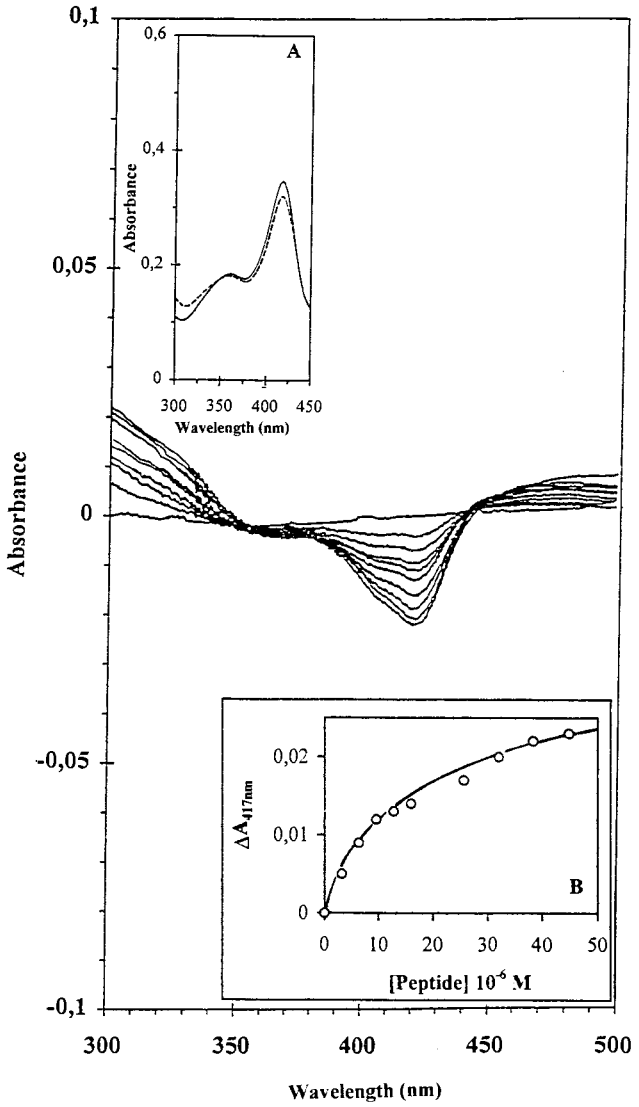

Figure 9. Difference spectra resulting from peptide−hemin-CN interactions. Conditions were the same as in Figure 8.

contrary to basic medium:

$$2 \text{ hemins} \rightarrow \text{dimer}$$

$$2 \text{ peptides} + \text{dimer} \rightarrow \text{peptide−dimer−peptide}$$

Since it was possible to study the equilibria of peptide−hemin associations in aqueous solution at acidic pH, we cannot affirm that the peptide−heme adducts obtained by the acetone method were representative of the associations existing in the heme enriched hemoglobin hydrolysate, although UV−visible spectra of heme were practically identical. Moreover, at high heme concentrations, heme−heme associations could lead to higher aggregation states than dimers both at acidic and at basic pH. In the same way, peptide−peptide associations could be involved for high peptide concentrations. Venkatesh et al. (1996) have reported a micellization process forming peptide aggregates at a critical micellar concentration of 9×10^{-5} M for a pentapeptide.

As a rule, the nature of the interactions involved in heme−peptide associations inside the heme enriched hemoglobin hydrolysate depends largely on the structure of each peptide. On one hand, ionized amino groups of the peptides could develop electrostatic interactions with the carboxylic groups of the propionic side chains of the heme. On the other hand, amino acids such as histidine, tyrosine, or cysteine could act as ligands toward heme-iron (Benson et al., 1995). Finally, peptides could bind heme through van der Waals and hydrophobic interactions between aromatic or saturated aliphatic groups and the macrocyclic core of heme (Leclerc et al., 1993; Venkatesh et al., 1996).

The peptide we have isolated was able to solubilize heme very efficiently, even at pH 2 where heme is totally insoluble. However, this peptide cannot develop efficient electrostatic interactions since at pH 2 it is positively charged and heme is neutral whereas, at pH 7.5, both heme and peptide are negatively charged. On the other hand, the lack of iron ligand on the side chains (His, Tyr, Cys, ...) makes unlikely ligancies. The study of the equilibrium between the peptide and heminCN (which is in the form of a monomer) by difference spectrometry allows us to confirm that heme−peptide associations did not occur through a direct interaction

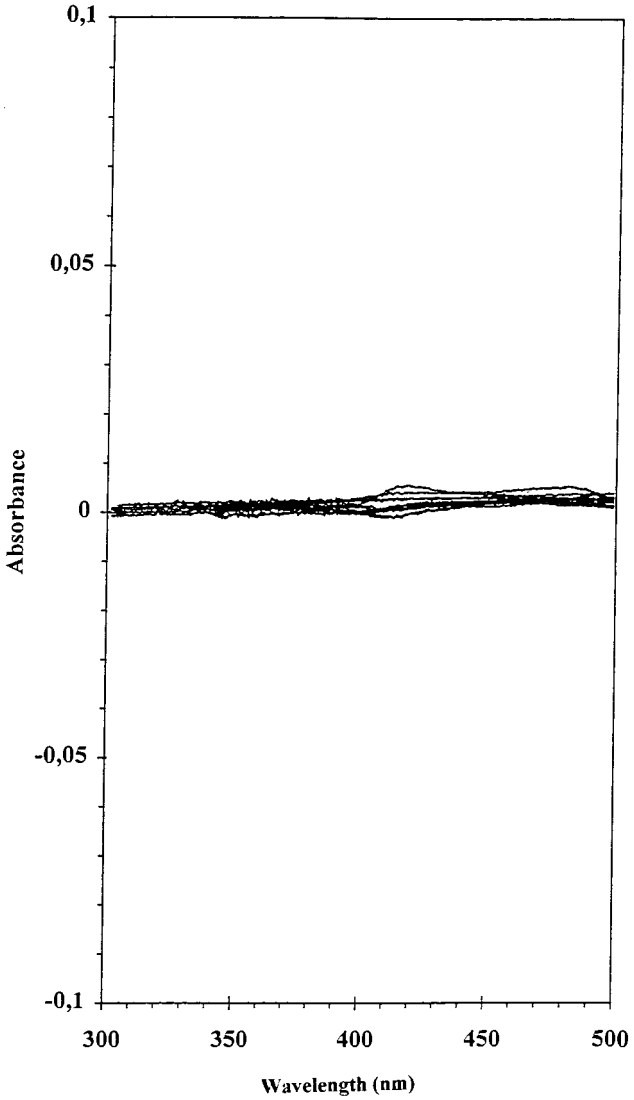

Figure 10. Difference spectra resulting from the addition of the peptide to hemin in the mixture of 50% Tris-HCl buffer and 50% ethylene glycol (v/v). Conditions were the same as in Figure 8.

with the iron. As a matter of fact, we can estimate with a good fitting that the dissociation constant of the peptide−heminCN complex would be 1.7×10^{-5} M (Figure 9, insert B). This value is practically identical to that of peptide−hemin complex (1.5×10^{-5} M) in which hemin is in the form of dimer. Moreover, we can exclude the displacement of a strong ligang as cyanide ion by a group from a side chain.

Both structural considerations and experimental results evidence the predominant role of the hydrophobic interactions in the formation of the heme−peptide complexes. The selected peptide with an average hydrophobicity of 905 cal/mol is an amphiphilic molecule allowing both hydrophobic interactions between apolar peptide groups and the tetrapyrolic macrocycle and interactions between water molecules and polar peptide groups. The position of this helical peptide in the hemoglobin conformation, which contracts interactions both toward the solvent and inside the protein (Figure 4), allows us to understand better its ability to solubilize hydrophobic molecules. The hydrophobic nature of these interactions is clearly demonstrated by the suppression of the heme−peptide interactions at pH 7.5 in the presence of ethylene glycol as shown by the difference spectra studies (Figure 10). Moreover the shift of the fluorescence emission maximum of the tryptophan toward shorter wavenlengths might indicate that this amino acid was involved in the hydrophobic associations.

CONCLUSION

In this study, we have described the water solubility of heme at acidic and basic pH in an heme enriched hemoglobin hydrolysate prepared by enzymatic hydrolysis and ultrafiltration at pilot plant scale. A pure peptide able to solubilize heme in the same conditions as the whole hemoglobin hydrolysate was isolated and taken as a model to investigate the mechanism of heme solubilization in such heterogeneous peptidic hydrolysates. We have proved that heme is mainly solubilized by hydrophobic interactions with the peptide and that ligancies or electrostatic interactions are not necessary. The stoichiometry of heme−peptide adducts depends on pH. At acidic pH for which heme alone is completely insoluble, the association between heme as dimer and two peptides seems likely whereas, at basic pH, heme dimer would be linked to one peptide. However, we cannot exclude the formation of high molecular weight aggregates or micelles, particulary for higher heme and peptide concentrations or in the whole heme enriched hemoglobin hydrolysate. Even if it is difficult to understand the heme−peptide stoichiometry in the whole hemoglobin hydrolysate, the UV−visible spectrophotometric study shows, as for the isolated peptide, that heme is in the form of dimer or higher state of aggregation. So, despite the high heme-iron−protein ratios and heme solubility of the heme enriched hemoglobin hydrolysates reported in the literature, the transport of heme as heme−heme adducts is not in favor of a good bioavailability of these preparations for treatment or prevention of iron deficiency because heme polymerization is known to decrease heme-iron intestinal absorption (Conrad et al., 1966; Reizenstein, 1980). Studies are now being carried out in our laboratory to prevent heme−heme adduct formation in the course of enymatic hydrolysis of bovine hemoglobin.

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