ISSN 0233-7657. Biopolymers and cell. 2006. Vol. 22. ISS 6. Translated from Ukrainia.

Physical factors of stability of triple helical collagen structures

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In the present study a complex approach of a number of experimental techniques, namely, infrared spectroscopy, quartz piezogravimetry, and differential scanning calorimetry, was used to estimate the hydration energy and enthalpy of interpeptide hydrogen bonds. On the basis of the results obtained the values of relative contributions of energies of interactions of different types into the total energy of stabilization of triple helical structures were evaluated.

Keywords: *collagen, poly(Gly-Pro-Pro), hydration energy, enthalpy of hydrogen bonds, infrared spectroscopy*

Introduction Fibrillar protein collagen is known to be the major structural component of connective tissue in mammals. Understanding of principles of structural organization of collagen and physical factors, which determine its stability, is an important and urgent task, since point mutations in genes, encoding amino acid sequence of collagen, often lead to changes in physical features of collagen fibrils and cause such serious diseases as Ehlers-Danlos' syndrome, Marfan's syndrome, *etc*.

The main structural units of collagen are rod-like molecules of tropocollagen in the shape of right-hand triple super-helices, composed of three primary left-hand polyproline II helices, built of repeated -Gly-*X*-*Y*- fragments, where *X* = Pro in 37.8% of cases, and *Y* = 4-Hyp in 28.8% of cases [1, 2].

Interpeptide hydrogen bonds, hydrogen bonds of C=O groups with water molecules, hydrophobic and Van der Waals interactions are considered to be major factors providing stability of collagen molecules.

According to the data of X-ray analysis (XRA), obtained on small fragments of native collagen molecules [3, 4] and model collagen peptides containing homotripeptide sites -Gly-Pro-Hyp- or -Gly-Pro-Pro- [5-7], the structure of hydrogen bonds in a triple helical molecule of tropocollagen is as follows: a network of interpeptide hydrogen bonds is formed between N_1H_1 groups of glycin residues of one chain and C_2O_2 groups of amino acid residues which are in *X* position in the neighbouring polypeptide chain; C_1O_1 groups of glycin bind to one water molecule, while C_3O_3 groups of amino acids in *Y* position bind to two water molecules. Moreover, polar side chains and NH-groups of amino acids also form hydrogen bonds with water, and a regular network of hydrogen bonds of N_2H_2 group of one chain and C_1O_1 groups of the neighbouring chain is formed in imino acid-deficient sites of collagen with the mediation of water molecules (Fig. 1).

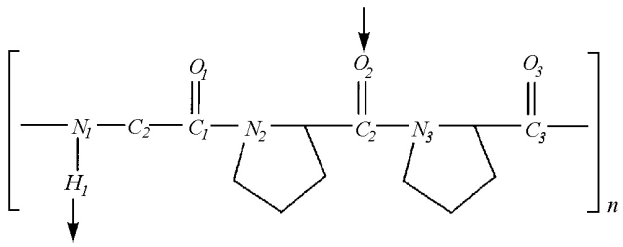

Fig.1. Structure of polytripeptide poly(Gly-Pro-Pro). CO and NH(N) groups of the repeated fragment -Gly-Pro-Pro- are designated in the following way: N_1H_1 and C_1O_1 belong to glycin; N_2 and C_2O_2 – to proline in *X* position; N_3 and C_3O_3 – to proline in *Y* position. Atoms, participating in the formation of interpeptide $N_1H_1\ldots C_2O_2$ hydrogen bond, are indicated by arrows.

According to the XRA data, in addition to the internal hydration shell, containing three water molecules per tripeptide in average and located in 2.7 Å from the nearest C=O groups of polypeptide, there is an external layer of hydration shell, whose molecules form hydrogen bonds with the water of the internal layer and are located in 3.6 Å from the tropocollagen molecule [5]. Similar data on the quantity of water molecules in the hydration shell of collagen proteins were obtained by calorimetry and gravimetry of collagen and theoretically calculated with the help of the Monte Carlo method [8, 9]. IR spectroscopy of the model collagen tripeptide $(Gly\text{-}Pro\text{-}Pro)_8$ revealed that the hydration process of collagen polypeptides has a multistage character and is accompanied by an isomorphic reorganization of the triple helical structure. The process of crystallization or ordering of the structure occurs after binding of 5-6 water molecules by each tripeptide [10].

Numerous works, devoted to the study on thermodynamic characteristics of collagen, have shown that the stability of triple helical collagen molecules is determined both by their amino acid content and a degree of hydration [11]. The enthalpy value of polytripeptide poly(Gly-Pro-Hyp) melting was found in [12] to exceed the enthalpy of polytripeptide poly(Gly-Pro-Pro) denaturation by ~2 times (3 and 1.77 kcal per mol per -Gly-X-Y- fragment, respectively) due to the difference in these two polytripeptides in an imino acid in *Y* position, though they have such common stabilizing factors as the presence of interpeptide hydrogen bonds and hydrogen bonds with water. Due to the formation of n- π^* bonds between oxygen and hydrogen of neighbouring peptide groups and *gauche*-effect [13, 14], the conformation of hydroxyproline is sterically more favorable for the formation of the triple helix and realization of contact Van der Waals interactions [15, 16]. Besides, OH group of hydroxyproline may form additional hydrogen bonds with water [17].

The amount of water, bound by collagen, changes the conditions of its denaturation considerably. Using differential scanning calorimetry (DSC) of native collagen [18] it was shown that at its maximally dehydrated state, corresponding to 0.07 mol water per mol -Gly-X-Y- the denaturation temperature takes maximal values (up to 210°C), while denaturation enthalpy has minimal values (0.7 kcal per mol -Gly-X-Y-). In a completely hydrated state the value of the denaturation temperature decreases to 41.1°C, while the denaturation enthalpy increases to 3.6 kcal per mol of -Gly-X-Y- fragment. The value of the denaturation enthalpy of dehydrated collagen is conditioned by the energetic cost of rupture of interpeptide hydrogen bonds which are kept in dehydrated collagen according to XRA [19]. High values of enthalpy of helix-coil transition are explained by the disruption of highly ordered hydration shell of collagen, which accompanies the rupture of interpeptide hydrogen bonds $N_1H_1\ldots C_2O_2$.

Therefore, obtained experimental data and theoretical models evidence to the important role of interpeptide hydrogen bonds and molecules of water in the formation of collagen structures. Nevertheless, the energies of interpeptide hydrogen bonds and energies of hydration of triple helical structures have not been determined quantitatively yet. These data would allow deeper understanding of the physical nature of stabilization of helical structures of the collagen type.

The aim of the current work was to evaluate the enthalpy of interpeptide hydrogen bonds, hydration energy in collagen and model collagen polytripeptide poly(Gly-Pro-Pro) as well as to determine the values of relative contributions of energies of interactions of different types into the total energy of stabilization of triple helical structures of the collagen type.

The evaluation was performed on the basis of data obtained by a complex of physical methods (IR

spectroscopy, piezogravimetry, and calorimetry). This approach was previously elaborated for the research on the process of hydration and structural transitions of nucleic acids [20, 21].

Materials and Methods The following substances were used in the work: type I collagen from calf skin, dried and acid-soluble (C9791, *Sigma*, USA), freeze-dried collagen from tail tendons of Wistar line mice, purified in the Biochemistry Department of Kharkiv National University according to a standard protocol [22], model collagen polytripeptide poly(Gly-Pro-Pro) (*Sigma*, P6665). Deuterium oxide (D_2O) with 99.84% isotope substitution was used to perform deutero-exchange.

Preparation of samples for IR spectroscopy and piezogravimetry. Polytripeptide poly(Gly-Pro-Pro) was dissolved in water at the concentration of 3.5 mg/ml. Collagen was dissolved in 0.01 molar solution of HCl and neutralized by adding a buffer mixture, containing hydroxymethylaminomethan, NaCl, KH_2PO_4/K_2HPO_4 buffer and H_2O. Collagen concentration in the final solution was 2.5 mg/ml, pH 7.45.

IR spectroscopy. Films of poly(Gly-Pro-Pro) and collagen, precipitated from the obtained solutions on the windows of CaF_2 served as samples for IR spectroscopy. To study hydration process of the films, the cuvette was completely dried by vacuum pumping, then moistened to the set values of relative humidity (RH) in the range of 0-86% by incubating the films in the vapors of saturated solutions of different salts in H_2O and D_2O [23]. IR spectra of dry and moistened films of investigated substances were recorded using two-ray spectrometer UR-20 (*Karl Zeiss, Jena*, Germany) with NaCl prism at room temperature. The spectrum was recorded in the frequency interval of 900-3700 cm^{-1}, which contains the absorption bands of the bound water and atomic groups of investigated substances, sensitive to hydration and structural state of collagen molecule [24].

Piezogravimetry. Hydration isotherms of poly(Gly-Pro-Pro) and collagen were obtained on the piezogravimetric equipment, described in [25]. In the beginning the films of poly(Gly-Pro-Pro) and collagen were completely dried, then they were moistened to the set values of RH in the range of 0-95%, by adding dosed water vapors. The experiment was performed at T = 20°C. Hydration isotherms were recorded in the form of dependence n(RH), where n is the amount of water, adsorbed by the sample (mol H_2O per mol of tripeptides of -Gly-X-Y- sorbent). The values of n at different RH levels were determined by the formula

$$n \quad \frac{M_{Gly\ X\ Y}}{M_{H_2O}} \quad \frac{f_i \quad f_m}{f_m} \qquad (1)$$

where $M_{Gly\text{-}X\text{-}Y}$ and M_{H2O} are the molecular masses of tripeptide -Gly-X-Y- and water, respectively; f_i is the difference in frequencies of reference and estimating quartz cavities at i humidity; f_m is the change of difference frequency, caused by a dry sample. The error was ±0.1 mol H_2O per 1 mol -Gly-X-Y-.

Calorimetry. The curves of heat absorption of dry and hydrated collagen samples from mouse tails were recorded using differential scanning microcalorimeter DSC-101 (*Setaram*, France). The details of experiments are described in [26].

Results and Discussion Fig. 2 presents the results of piezogravimetry of polytripeptide poly(Gly-Pro-Pro) and collagen. Curve *1* (Fig. 2, *a, b*) shows the hydration isotherm. The level of water sorption at the same RH values is a little higher in collagen than in the polytripeptide.

To perform a more detailed analysis of the results of piezogravimetry, the experimental isotherms of hydration were approximated using modified d'Arcy and Watt equation [25], which allows taking into consideration the heterogeneity of adsorbent:

$$V(i) \quad \frac{V_m \quad a_L \quad i}{1 \quad a_L \quad i} \quad a_H \quad i \quad \frac{b \quad i}{1 \quad b \quad i} \qquad (2)$$

where i is the relative humidity; 1st summand describes the stage of absorption of water molecules according to Langmuir law; 2nd shows the absorption of water molecules according to Henry law and 3rd one describes the multilayer absorption; V_m is the monolayer capacity; a_L, a_H, b are activities of water on the stages of Langmuir, Henry, and multilayer absorption, respectively.

Decomposition results are presented in Fig.2, *a, b* (curves 2-4). The parameters of d'Arcy and Watt equation, found in the decomposition process, are presented in Table 1.

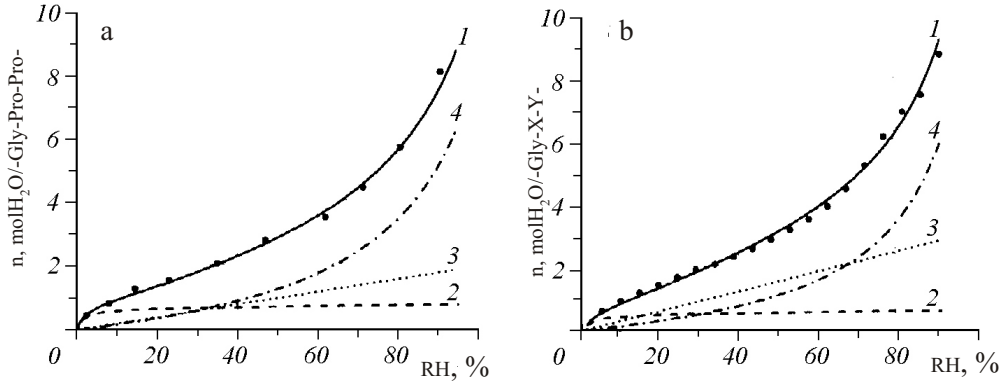

Fig.2. Results of piezogravimetry of poly(Gly-Pro-Pro) (*a*) and collagen (*b*): isotherms of hydration (*1*), curves of Langmuir (*2*), Henry (*3*) and multilayer (*4*) absorption, obtained after decomposition of isotherms, using the modified d'Arcy and Watt

Table 1

Parameters of modified d'Arcy and Watt equation, obtained after decomposition of hydration isotherms of poly(Gly-Pro-Pro) and collagen

Substance	V_m	a_L	a_H	b
Poly(Gly-Pro-Pro)	0.81±0.05	39.9±0.02	2.01±0.05	0.89±0.02
Collagen	0.52±0.05	43.2±0.02	2.98±0.05	0.89±0.02

Note: V_m is the capacity of monolayer; a_L, a_H, and *b* are the activities of water on the stages of Langmuir, Henry, and multilayer absorption, respectively.

Fig. 2, a, b shows that at 90% RH the first hydration layer of polypeptide and collagen, where water molecules are adsorbed according to Langmuir and Henry laws, has 2.5±0.1 and 3.2±0.1 water molecules, respectively. These water molecules form the most stable hydrogen bonds with hydration centers of the investigated substances.

Using the parameters of the modified d'Arcy and Watt equation, obtained at decomposition of hydration isotherms, it is possible to find absorption heat $Q=R\,T\,ln(K_i/K_L)+T\,S_i$ for absorption layers of Langmuir and Henry (Q_L and Q_H), respectively, as well as enthalpy and energy of hydration $H_{hydr}= E_{hydr}=- Q_i$ poly(Gly-Pro-Pro) and collagen. The constants of absorption equilibrium of Langmuir, Henry and polymolecular absorption in the reaction [vapour=substanceљsingle complex], K_i may be found according to the formula $K_i = a\,K_w$, where K_w is the constant of water condensation [saturated vapour liquid]. It is noteworthy that according to this estimating method the values of Q_i in each absorption layer *i* are the difference between total absorption heat of water-adsorbent and the heat of water molecules condensation. Therefore, the values of H_{hydr} and E_{hydr} are also difference (excess) enthalpy and energy of hydration, respectively.

Unfortunately, the approach that we use does not allow estimating the change in the system entropy S_i caused by interaction of water molecules with polymer molecule. However, according to [27], its value is close to zero. While determining the total heat of water absorption, the contribution of polymolecular absorption was not taken into account, as the constants of absorption *b* for collagen and poly(Gly-Pro-Pro) are close to 1.

The values of absorption heat and total enthalpy of hydration of polytripeptide and collagen are presented in Table 2.

The absolute values of the total enthalpy of hydration of collagen H_{hydr} is 1.12 higher that that of polytripeptide. At the same time, Q_L values of investigated samples do not differ significantly, and Q_H of collagen is 1.6 higher than that of polytripeptide. The differences in hydration of samples may be connected with the increase in the number of hydrophilic sites in collagen caused by substitution of some imino acids in *X* and *Y* positions by amino acids. Additional hydration centers may also be formed by hydrophilic amino acid radicals and N-H groups of the peptide groups of amino acids.

Taking into consideration the data, concerning the quantity of water molecules in the internal layer of hydration shell of samples obtained by piezogravimetry, the average enthalpy of peptide-water hydrogen bonds in poly(Gly-Pro-Pro) and collagen can be estimated as – 4.3 and – 3.9 kJ/mol, respectively.

Table 2
Absorption heat of water (kJ/mol of -Gly-X-Y- fragment) according to the laws of Langmuir and Henry, Q_L and Q_H, respectively

Substance	$ДQ_L$	$ДQ_H$	$ДH_{hydr}$
Poly(Gly-Pro -Pro)	9.2±0.02	1.7±0.01	-10.9±0.04
Collagen	9.5±0.02	2.7±0.01	-12.2±0.04

We compared the obtained values of total enthalpy of hydration of collagen and poly(Gly-Pro-Pro) with some known reference data. In [28] the estimated values of free energy of C=O…H-O-H hydrogen bonds in N-methylacetamide and water-water hydrogen bonds are –31.78 and –27.59 kJ/mol, respectively. Therefore, the value of difference hydration energy is –4.19 kJ which is close to the values, obtained by us.

To perform a detailed research on hydration of poly(Gly-Pro-Pro) and collagen as well as on structural transitions, accompanying this process, IR spectroscopic study of sample films in the wide range of RH values was conducted. Obtained IR spectra are presented in Fig. 3. Spectra analysis revealed that the formation of hydration shell of investigated substances, occurring with RH increase, was accompanied by considerable changes in spectral parameters of the main absorption bands.

Detailed spectra analysis was performed by building the curves of dependencies of frequencies ν (cm^{-1}) of the main Amide bands from RH (%) and n, where n is the quantity of water molecules, adsorbed by one -Gly-X-Y- fragment; n was determined on the basis of hydration isotherms (see above). The curves of ν(RH) and $\nu(n)$ were built for Amide I, Amide II, and Amide II' absorption bands (Fig.4). Atom groups, whose vibrations contribute to these bands, according to XRA of model collagen structures, participate in the formation of interpeptide hydrogen bonds and hydrogen bonds with water molecules [3, 5, 6]. Since absorption bands caused by bending vibrations of OH-groups of water molecules, overlap with the Amid I band of non-deuterated proteins, the analysis of Amid I band was performed for deuterated samples. The analysis of ν(RH) and $\nu(n)$ dependencies for Amid I, Amid II, and Amid II' of poly(Gly-Pro-Pro) and collagen is presented further.

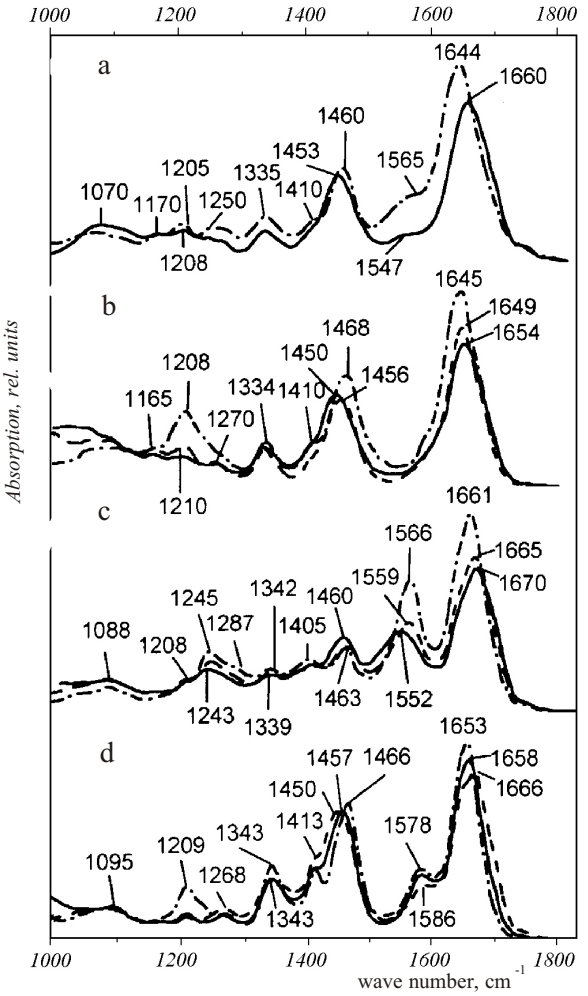

Fig.3. IR spectra of dry and moistened films of samples in the atmosphere of light and heavy water: *a* – poly(Gly-Pro-Pro) in H_2O: *b* – poly(Gly-Pro-Pro) in D_2O; *c* – collagen in H_2O; *d* – collagen in D_2O; solid line corresponds to 0% RH, dashed line – 15% RH, chain line – 76% RH

The hydration of the sample results in a low frequency shift of Amid I band (Fig. 4, *a, d*), the maximum values of which ν = –9 cm^{-1} for poly(Gly-Pro-Pro) and ν = –13 cm^{-1} for collagen are achieved after absorption of three water molecules by each -Gly-X-Y- fragment. It is accompanied by an increase of the Amide I band intensity (Fig. 3, *b, d*). Taking into consideration XRA [3, 5, 6], these spectral changes may be connected with the formation of interpeptide hydrogen bonds and H-bonds of carbonyl groups of glycine C_1O_1 and proline C_3O_3 with water molecules (Fig. 1). The increase of the sample hydration level also results in high frequency shifts of

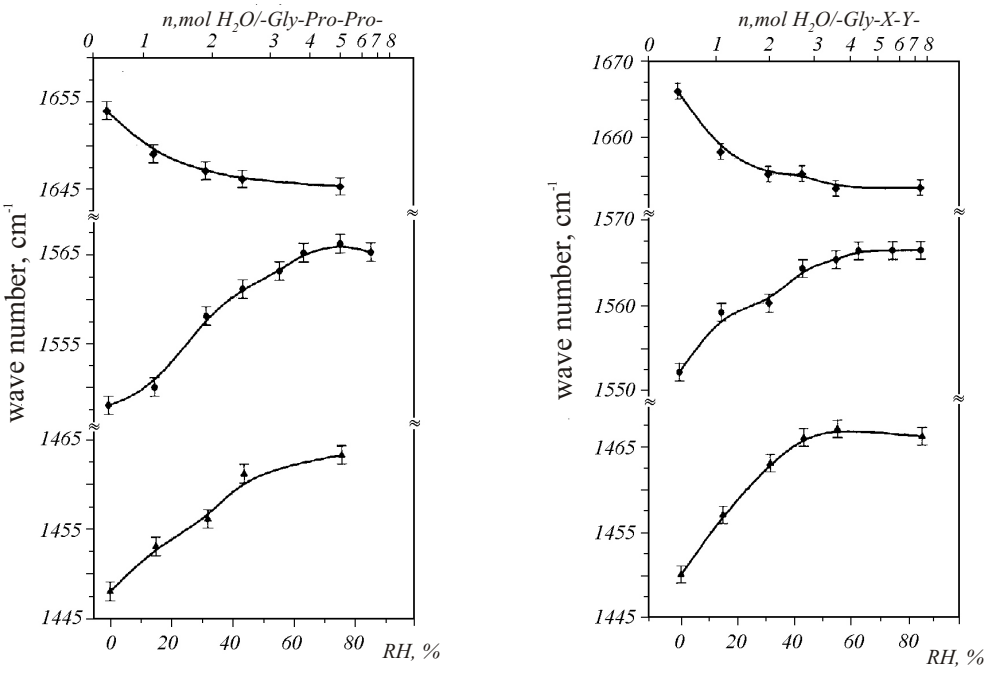

Fig.4. Frequencies of Amid I, Amid II, Amid II' bands of polytripeptide poly(Gly-Pro-Pro) (*a, b, c*) and collagen (*d, e, f*) in the conditions of different values of relative humidity in the atmosphere of H_2O and D_2O. Abscissa axes represent the hydration level of the sample: the higher axis (*n*) shows the number of mol of water, adsorbed by one mol of tripeptide -Gly-X-Y-, the lower axis (RH%) is the relative humidity. Dependence *n*(RH) was found on the basis of hydration isotherms.

Amid II and Amid II' absorption bands, having the values of $\nu = 17$ and 18 cm^{-1} in poly(Gly-Pro-Pro) and $\nu = 14$ and 16 cm^{-1} in collagen (Fig. 4, *b, c, d, e*). Similarly to the Amid I band, the saturation of frequency shifts of these bands takes place at the absorption of the first three water molecules by each -Gly-Pro-Pro- fragment of polytripeptide and four water molecules by each -Gly-X-Y- fragment of collagen.

High frequency shift of absorption bands, assigned to bending vibrations of atoms, is known to occur at formation of hydrogen bonds by these atom groups. The main contribution into absorption in Amid II (Amid II') area is made by bending vibrations of NH (ND) groups [29, 30]. In case of poly(Gly-Pro-Pro) such groups are present only in the peptide groups of glycin, as proline is an imino acid. According to the XRA data, interpeptide $N_1H_1\ldots C_2O_2$ hydrogen bonds are kept in dry poly(Gly-Pro-Pro) [31], therefore, high frequency shift of Amid II band, evident at poly(Gly-Pro-Pro) moistening, may be connected with the strengthening of $N_1H_1(N_1D_1)\ldots C_2O_2$ interpeptide hydrogen bonds which accompanies structural reorganization of the triple helix at the formation of hydrate structure of polytripeptide. Similar phenomenon of strengthening the net of interpeptide bonds in polypeptide $(Gly\text{-}Pro\text{-}Pro)_8$ at hydration is described in [24].

In case of collagen, both the vibrations of N-H groups of glycine and amino acids, substituting proline and hydroxyproline in *X* and *Y* positions contribute to the absorption area of Amid II, therefore, high frequency shift of Amid II band, evident at moistening, should be connected not only with the strengthening of interpeptide hydrogen bond, but also with the formation of hydrogen bonds of N-H-groups of amino acids with water molecules. We also assume that low frequency shifts of Amid I band at the hydration of collagen and poly(Gly-Pro-Pro) reflects not only the formation of hydrogen bonds of carbonyl groups with water, but also the strengthening of interpeptide hydrogen bonds.

Thus, the changes in IR spectrum of poly(Gly-Pro-Pro) and collagen, observed at hydration, allow making the following conclusions: 1) in the course of hydration of the sample 3-4 water molecules per each -Gly-X-Y- fragment form the most stable hydrogen bonds with C=O groups of polytripeptide; probably, they constitute the internal layer of hydrate shell of polytripeptide; 2) hydration of the sample is

accompanied by structural reorganization of the triple helix and strengthening of interpeptide hydrogen bond.

To determine the influence of hydration on the conformational state of triple helical collagen molecule, the values of *R* ratio was calculated as a relation of peak intensities of Amid III and 1450 cm^{-1} absorption bands were estimated at different RH degree. It is known that for native collagen *R 1.38 [32]*. Obtained dependence *R(n)* is presented in Fig. 5. Ratio *R* reached 1.38 at RH 70%, that corresponds to the absorption of 4-5 water molecules per -Gly-X-Y- fragment. It shows that at RH 70% the conformational shift of collagen caused by its hydration is completed and the collagen molecules obtain their native triple helical structure.

Taking into account the data of IR spectroscopy, piezogravimetry, DSC and vibrational analysis of Amid I band of collagen proteins, performed previously with the consideration of resonance of interactions of carbonyl vibrations [33, 34], it is possible to estimate the enthalpy of interpeptide hydrogen bonds N_1-H_1…O_2=C_2 in poly(Gly-Pro-Pro) and collagen. We used several estimating approaches combining the data of different methods. The results are presented below.

1. The enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in poly(Gly-Pro-Pro) may be estimated theoretically on the basis of the data on frequency shift of the vibration of the corresponding carbonyl group at its transition from the free state into the hydrogen-bound state, using the empirical dependency of enthalpy of hydrogen bond $H_{\text{H-bond}}$, formed by carbonyl group, on $\nu_{\text{H-bond}}$, frequency shift of C=O group oscillation at the formation of the corresponding H-bond:

$$H_{\text{H-bond}} = C \quad \nu_{\text{H-bond}} \text{ (kJ/mol)}, \quad (3)$$

where C = 0.205 kJ cm^{-1} mol^{-1}.

The value of frequency shift of stretching vibrations of carbonyl group C_2O_2 due to the formation of interpeptide hydrogen bond was estimated by us in [33] with the consideration of resonance interactions of carbonyl vibrations: $\nu_{\text{H-pept}}$ = -37cm^{-1}. According to equation (3), the enthalpy of hydrogen bond N_1-H_1…O_2=C_2 $H_{\text{H-pept}}$ = -7.6±1.0 kJ/mol corresponds to this value.

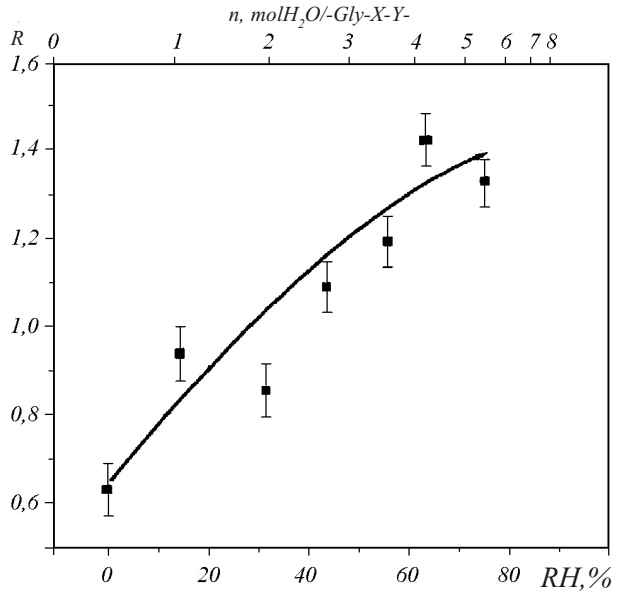

Fig.5. Ratio *R(n)* of peak intensities of Amid III and 1450 cm^{-1} absorption bands at different RH values. Abscissa axes present hydration level of the sample: the higher axis (*n*) shows the number of mol of water, adsorbed by one mol of tripeptide -Gly-X-Y-, the lower axis (RH%) is the relative humidity. Dependence *n*(RH) was found on the basis of hydration isotherms.

The enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in poly(Gly-Pro-Pro) may be also found using the results of IR spectroscopy. For this reason we need to present the total enthalpy of hydrogen bond N_1-H_1…O_2=C_2 as $H_{\text{H-pept}} = H_{\text{dry}} + H_{\text{dry wet}}$, where H_{dry} and $H_{\text{dry wet}}$ are the enthalpy of hydrogen bond N_1-H_1…O_2=C_2 in dry polytripeptide and the value of this enthalpy increase at the conformational transition, that accompanies the hydration of the sample, respectively.

H_{dry} value may be determined from the formula (3), where $\nu_{\text{H-pept}} = \nu_{\text{dry}} = \nu_0 - \nu_{\text{dry}}$ is the shift between the frequency of C_2O_2 group vibration in a free state and in dehydrated triple helical poly(Gly-Pro-Pro), ν_0 = 1693 cm^{-1} and ν_{dry} = 1664±2 cm^{-1}, respectively [16]. Therefore, we obtain ν_{dry} = -28±2 cm^{-1} and H_{dry} = -5.74±0.4 kJ/mol. Frequency shift of the maximum of Amid I band at hydration is $\nu_{\text{dry wet}}$ = -9 cm^{-1} (Fig. 4, *d*), $H_{\text{dry wet}}$ = -1.84±0.2 kJ/mol by equation (3). Thus, the total energy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in poly(Gly-Pro-Pro) is $H_{\text{H-pept}}$ = -7.58±1.2 kJ/mol. This result is close to the theoretically calculated value.

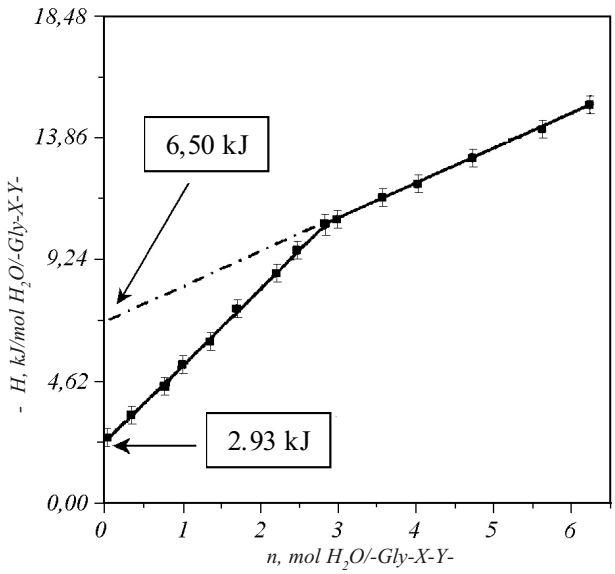

Fig.6. Dependences of enthalpy of collagen melting H on its hydration degree n, where n is a number of mol of water, adsorbed by one mol of tripeptide -Gly-X-Y- [26]).

2. The enthalpy of interpeptide hydrogen bond in collagen can also be estimated on the basis of IR spectroscopy data and the dependence of enthalpy of collagen melting H on the degree of its hydration, obtained previously using DSC and published in [26]. The dependence $H(n)$, where n is the quantity of water mol per mol of -Gly-Pro-Pro- fragment, is presented in Fig. 6.

The total enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in collagen as well as in poly(Gly-Pro-Pro) may be presented in the form of the following sum: $H_{\text{H-pept}} = H_{\text{dry}} + H_{\text{dry wet}}$. The value H_{dry} may be found from the $H(n)$ curve, where $H_{\text{dry}} = H(0) = -2.93\pm0.4$ kJ/mol. Frequency shift of the maximum of Amid I band at hydration is $\nu_{\text{dry wet}} = -13$ cm^{-1} (Fig. 4, *a*), *i.e.* $H_{\text{dry wet}} = -2.67\pm0.2$ kJ/mol. The calculated value of the total enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in collagen may be somewhat understated, due to a possible formation of new N-H…O=C hydrogen bonds in the random coil collagen. This process is exothermal and causes the decrease in enthalpy value of helix-ball transition, estimated calorimetrically. That is why we consider the value $H_{\text{H-pept}} = -5.6\pm1.2$ kJ/mol to be a lower threshold of the absolute value of total enthalpy of interpeptide hydrogen bond N_1-H_1...O_2=C_2 of collagen.

To determine an upper threshold of the absolute value of total enthalpy of interpeptide hydrogen bond N_1-H_1...O_2=C_2 of collagen, the values of H of $H(n)$ curve in each point will be presented as a sum $H(n) = H_{\text{H-pept}}(n) + H_{\text{H-wat}}(n)$, where the values $H_{\text{H-pept}}(n)$ and $H_{\text{H-wat}}(n)$ correspond to the enthalpy of interpeptide hydrogen bond N_1-H_1...O_2=C_2 and the enthalpy of peptide-water hydrogen bonds. Non-monotonous behavior of $H(n)$ curve is explained by the fact that at the initial stage of hydration process in 0 n 3 area there is simultaneous formation of hydrogen bonds with water molecules of the internal hydration hydrate shell and strengthening of interpeptide hydrogen bonds. In $n > 3$ area the value $H_{\text{H-pept}}(n)$ *const* and the increase of total H is mainly connected with the increase in quantity of water molecules, adsorbed in the external layer of hydration shell.

Taking the abovementioned into account we determined the average value of the increase in total enthalpy of denaturation caused by sorption of a single water molecule of the external hydration shell and found it to equal -1.36 kcal/mol. According to the piezogravimetry data, the average energy of hydration for water molecules of the internal layer exceeds that of water of the external layer of the hydration shell, therefore, the following correlation is correct for n>3 interval: $H_{\text{H-pept}} > H(n) - n$ (-1.36) kJ/mol. Taking the value of $H(n)$ at any n>3 it is evident that the upper threshold of the absolute value of total enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 equals $H_{\text{H-pept}} < -6.5$ kJ/mol.

Therefore, we determined both upper and lower thresholds of the value of total enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 of collagen, the average value of this enthalpy is -6.0±0.5 kJ/mol. The average value $H_{\text{H-pept}}$ for poly(Gly-Pro-Pro) is -7.6±0.2 kJ/mol.

Obtained values of enthalpy of interpeptide hydrogen bond N_1-H_1…O_2=C_2 in collagen structures are in good agreement with the values of experimentally determined in [36] energy of N-H…O=C hydrogen bonds in proteins $E_{\text{H-pept}} = -6.3$ kJ/mol and the energy of hydrogen bonds N_1-H_1…O_2=C_2 in model collagen peptide $(\text{Gly-Pro-Hyp})_8$, $E_{\text{H-pept}} = -6.2$ kJ/mol, obtained recently using the molecular dynamics method [37].

The estimation of percent contributions of energies of different types of interactions into the total energy of

molecule stabilization is widely used to describe physical basics of biological macromolecules stabilization. Usually these percent contributions are calculated according to equation

$$H_{trans} = H_{H\text{-}pept} + H_{hydr} + H_{other}, \quad (4)$$

where H_{trans} is the enthalpy of helix-coil transition, determined calorimetrically; $H_{H\text{-}pept}$, H_{hydr}, H_{other} are the enthalpy contributions of interpeptide hydrogen bonds, hydrogen bonds with water and interactions of other types (Van der Waals interactions, hydrophobic interactions, *etc*.) [21].

However, after setting our calculated values $H_{H\text{-}pept}$ and H_{hydr} into the equation (4), we obtain $H_{trans} \approx H_{H\text{-}pept} + H_{hydr}$ for collagen, and $H_{trans} > H_{H\text{-}pept} + H_{hydr}$ for poly(Gly-Pro-Pro). This correlation may be due to the fact that equation (4) does not include the term describing the exothermic process of aggregation of denaturated polypeptide chains that results in formation of new interpeptide hydrogen bonds [38]. Aggregation degree increases with the increase in protein concentration in the solution. Therefore, considering the possibility of aggregation, the equation (4) should be changed in the following way: $H_{trans} = H_{H\text{-}pept} + H_{hydr} + H_{other} + H_{aggr}$, where H_{aggr} – enthalpy of aggregates formation.

Taking into account that H_{other} and H_{aggr} were not calculated in this work, we did not estimate percent contributions of $H_{H\text{-}pept}$, H_{hydr}, and H_{other}, however, it was possible to calculate the correlation of $H_{H\text{-}pept}$: H_{hydr} contributions, which is 1:2 for collagen and 1:1.4 for poly(Gly-Pro-Pro). These values are lower than the average correlation $H_{H\text{-}bond}$: H_{hydr} = 1:2.3, obtained for different types of nucleic acids [21], where H_{bond} is the enthalpy of hydrogen bonds in AT and GC pairs. It evidences to a more considerable contribution of energy of hydrogen bonds between different polymer chains into the total energy of stabilization of triple collagen helix in comparison with the double helix of DNA. The question of energy contribution of other types of interaction requires further research.

Conclusions The binding of 3-4 water molecules of the internal hydration shell per one -Gly-X-Y- fragment takes place at hydration of poly(Gly-Pro-Pro) and collagen in 0-60% RH interval. This process is accompanied by conformational transition of the triple helix and strengthening of interpeptide N_1-H_1…O_2=C_2 hydrogen bonds. Hydration energies of poly(Gly-Pro-Pro) and collagen are -10.9 and -12.2 kJ/mol, respectively. The enthalpy of interpeptide N_1-H_1…O_2=C_2 hydrogen bonds for poly(Gly-Pro-Pro) and collagen equals -7.6±0.2 and -6.0±0.5 kJ/mol, respectively. The correlations of $H_{H\text{-}pept}$: H_{hydr} contributions are 1:2 in collagen and 1:1.4 in poly(Gly-Pro-Pro).

The authors express their sincere gratitude to Professor E. E. Persky (KhNU) for his interest to this work and discussion of results.

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Физические факторы стабильности трехспиральных структур коллагенового типа

Резюме

Вычислены энтальпия межпептидных водородных связей и энергия гидратации коллагена, а также модельного коллагенового политрипептида poly(Gly-Pro-Pro) и определены относительные вклады энергий взаимодействий различных типов в общую энергию стабилизации трехспиральных структур коллагенового типа. Расчет проводили на основании данных комплекса экспериментальных физических методов: ИК спектроскопии, пьезогравиметрии и дифференциальной сканирующей калориметрии.

Ключевые слова: коллаген, poly(Gly-Pro-Pro), энергия гидратации, энтальпия водородных связей, ИК спектроскопия.

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УДК 577.322
Надійшла до редакції 03.04.06