

*Biophysics*

## Molecular Mechanism for the Phylogenetic Change of Thermostability of Fibril-Forming Collagen. Evidence that (Gly-X-Hyp) Triplets are Main Factors Determining Stability of Collagen

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**ABSTRACT.** Published data relating the concentration of 4-hydroxyproline residues to the stability of collagen was used to estimate the different effects of two types of triplets: (Gly-X-Hyp) and (Gly-Pro-Hyp). The data comprised mainly fibril-forming collagens, which exhibit highly cooperative helix-coil transitions at temperatures that are remarkably close to body temperature. A wide range of denaturation temperatures was therefore ensured by including collagen from different species of fish, including some living in the arctic, amphibians and mammals. As a first approximation, the dependence of the denaturation temperature,  $T_d$ , of collagen on hydroxyproline content was considered as a linear function:

$$T_d = T_0 + K_1 N_{(GXO)} + K_2 N_{(GPO)},$$

where the coefficients  $K_1$  and  $K_2$  characterize the degree of influence of the two types of triplet. Regression analyses showed that  $K_1$  exceeded  $K_2$  by about 10 times. Analysis of the dependence of the enthalpy of denaturation on hydroxyproline content also showed that triplets of the type (Gly-X-Hyp) containing water had a larger effect than (Gly-Pro-Hyp) triplets and that the primary factor determining increased stability was the concentration of (Gly-X-Hyp) triplets, while (Gly-Pro-Hyp) triplets governed the nonlinear character of the relationship. We conclude that the main factor stabilizing the collagen triple helix and governing the phylogenetic change of collagen thermostability is the concentration of triplets of the type (Gly-X-Hyp). © 2010 Bull. Georg. Natl. Acad. Sci.

**Key words:** collagen, phylogenesis, thermostability, water-bridge structure.

### INTRODUCTION

Collagen, the major component of connective tissues, has a specific triple-helical conformation. The characteristic feature of this structure is the strictly repeated amino-acid sequence: (Gly-X-Y), where X and Y can be almost any amino acid, but most frequently they are proline (Pro) in the X position and hydroxyproline (Hyp) in the Y position [1, 2]. The most generally accepted water-bridged structures [3,4] stabilizing the structure are sequence-de-

pendent, in that they require the participation of the side chain OH-group of hydroxyproline in the Y position on one chain and a residue other than proline in the X position on the adjacent chain. The sequence is thus crucial in determining the role of water in structure stabilization.

The triple-helical structure of collagen is maintained over a wide range of animal phyla, and its denaturation temperature appears to be correlated with the upper limit of environmental temperature of the host organism (Fig.

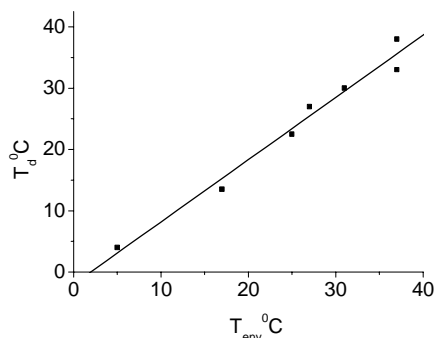


Fig. 1. Dependence of the denaturation temperature ( $T_d$ ) on environment temperature ( $T_{env}$ ) for fibril-forming collagens of various origin.

1.), if it is cold-blooded [5-7]. Analysis of the stability of collagens of widely diverse origins, performed in our earlier papers, showed that for fibril-forming collagens the denaturation temperature  $T_d$  appears to be determined by the concentration of 4-hydroxyproline localized in the third position of a triplet, while 3- and 4-hydroxyproline in the second position do not participate in stabilization [8,9]. However, some collagen-like peptides with these residues in the X position may form a triple helix [10]. As 4-hydroxyproline is localized almost exclusively in the Y position in collagen, as an approximation it may be considered to exist in only two types of triplets: (Gly-X-Hyp) and (Gly-Pro-Hyp). As these differ from each other in their thermodynamic and structural characteristics [11-14], it seems possible that one of the causes of the nonlinear dependence of collagen thermostability on hydroxyproline content may be the different influence of these triplets on the temperature (Fig. 2) and enthalpy denaturation (Fig. 3). In this paper we make separate estimations of the contributions of (Gly-Pro-Hyp) and (Gly-X-Hyp) triplets to the phylogenetic change of collagen thermostability, attempting to answer the question: *what type of triplet plays the principal role in determining increased thermostability and what kind determines its nonlinear character.*

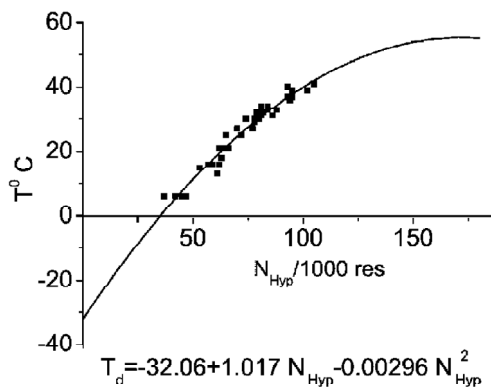


Fig. 2. Dependence of the denaturation temperature ( $T_d$ ) of various collagens [8,18] on 4-Hyp content.

## RESULTS OF THE ANALYSIS

### 1. Temperature of denaturation: non-linear regression analysis

When the denaturation temperature of fibril-forming collagens was plotted against their hydroxyproline concentration, the data showed a slight downward curving non-linearity (see [8], Fig.2). Limiting a general polynomial dependence to the first three terms, the temperature of denaturation was written as:

$$T_d = T_0 + A_1 N_{Hyp} + B_1 (N_{Hyp})^2 \quad (1)$$

Here,  $N_{Hyp}$  is the number of hydroxyproline residues per 1000 residues and the numerical values of coefficients  $A_1$  and  $B_1$  were found by regression analysis. The fibril-forming collagens yielded:

$$T_d \text{ } ^\circ\text{C} = (-33.95 \pm 4.46) + (1.076 \pm 0.12)N_{Hyp} - (0.0034 \pm 0.0008)N_{Hyp}^2 \quad (2)$$

The underlying cause of the nonlinearity was revealed by examining the different effects of the (Gly-X-Hyp) and (Gly-Pro-Hyp) triplets. As a first approximation we assume that they affect  $T_d$  independently and linearly. The denaturation temperature is therefore given by:

$$T_d = T_0 + K_1 N_{(GXO)} + K_2 N_{(GPO)} \quad (3)$$

here,  $N_{(GXO)}$  and  $N_{(GPO)}$  are the number of (Gly-X-Hyp) and (Gly-Pro-Hyp) triplets per 1000 residues, and coefficients  $K_1$  and  $K_2$  characterize the degree to which corresponding triplets influence the denaturation temperature. Nonlinear regression analysis of the data revealed how (Gly-X-Hyp) and (Gly-Pro-Hyp) concentrations increased with hydroxyproline. With increasing hydroxyproline content (Gly-Pro-Hyp) curved upwards (Fig. 4) and (Gly-X-Hyp) curved downwards (Fig.5). Algebraically:

$$N_{GPO} = C + A_2 N_{Hyp} + B_2 N_{Hyp}^2 \quad (4)$$

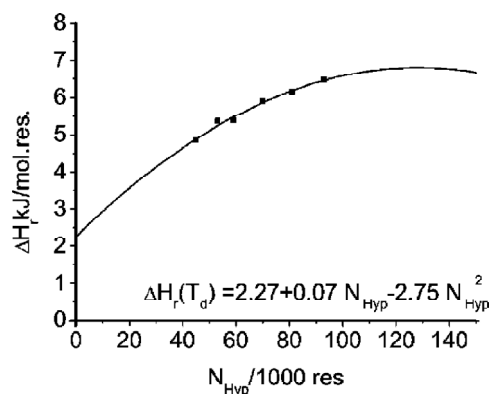


Fig. 3. Dependence of the standard denaturation enthalpy at 25 $^\circ$ C of various collagen differing by hydroxyproline concentration. The data taken from Table 3.

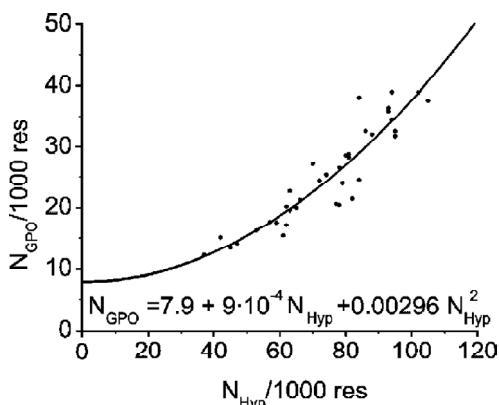


Fig. 4. Dependence of the estimated number of Gly-Pro-Hyp triplets in various collagens on hydroxyproline content

$$N_{GPO} = N_{Hyp} - N_{GPO} = -C + (1 - A_2)N_{Hyp} - B_2 N_{Hyp}^2 \quad (5)$$

and least squares regression yielded:

$$C = 9.18 \pm 7.85; A_2 = -0.043 \pm 0.225; B_2 = 0.00329 \pm 0.00156. \quad (6)$$

By substituting Eq. (5) and Eq. (6) into Eq. (4) and comparing the coefficients with those of Eq. (2), we find:

$$K_1 + (K_2 - K_1)A_2 = A_1; (K_2 - K_1)B_2 = B_1. \quad (7)$$

Thus we estimate that  $K_1 = 1.03 \pm 0.26$ ;  $K_2 = 0 \pm 0.59$ . A more precise difference between the values of these coefficients can be found directly from the relation:  $K_1 - K_2 = 1.03 \pm 0.54$ . Thus the difference between these coefficients is twice as large as the standard error and the probability that  $K_1 > K_2$  is about 95%. From equation (3) one can calculate that the maximum denaturation temperature occurs when  $N_{Hyp}$  satisfies:

$$(dT_d/dN_{Hyp})_{max} = |1.0438 - 0.00674N_{Hyp}| = 0; N_{Hyp} = 155 \text{ residues / 1000 residues}. \quad (8)$$

The maximum temperature at this concentration of hydroxyproline is equal to  $T_d = 49.2^\circ\text{C}$ . Thus we conclude that the water-bridged structure of fibril-forming collagens will limit the habitat temperature of animals because of its structural features. It will be shown below that the same value of hydroxyproline marks a maximum in denaturation enthalpy.

Table 1

Magnitudes  $K_1$  and  $K_2$  estimated from equations

EQUATION	$T_0$ ( $^\circ\text{C}$ )	$K_1$ ( $^\circ\text{C res}/1000 \text{ res}$ )	$K_2$ ( $^\circ\text{C res}/1000 \text{ res}$ )	$K_1/K_2$	$\sigma = \sqrt{\sum(T(i)^{obs} - T(i)^{cal})^2 / n - 1}$ ( $^\circ\text{C}$ )
$T_d = T_0 + K_1(\text{Gly-X-Hyp}) + K_2(\text{Gly-Pro-Hyp})$	-33.95	$1.16 \pm 0.0061$	$0.091 \pm 0.0085$	$12.62 \pm 1.102$	4.79
$T_d = T_0 + K_1(\text{Gly-X-Hyp}) + K_2(\text{Gly-Pro-Hyp})$ *	$-18.49 \pm 0.81$	$0.87 \pm 0.0168$	$0.091 \pm 0.0085$	$9.64 \pm 0.715$	2.75

\*  $T_0$ ,  $K_1$  and  $K_2$  were calculated by multiple regression analysis.

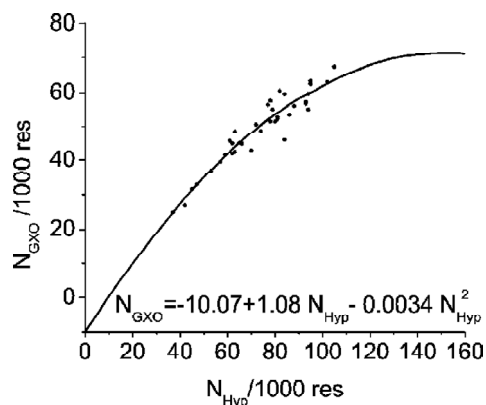


Fig. 5. Dependence of estimated number of Gly-X-Hyp triplets in various collagens on hydroxyproline content

## 2. Multiplet regression analysis.

The nonlinear regression analysis of the previous section showed that (Gly-X-Hyp) triplets were more stabilizing than (Gly-Pro-Hyp). However, in order to find out more precisely the magnitude of this effect, least square multiple regression analysis of the data was undertaken using Eq. (4). The analysis was carried out in two ways: 1) the constant term ( $T_0$ ) was calculated from the dependence of denaturation temperature on hydroxyproline concentration. This reduced the degrees of freedom and it was then only necessary to calculate  $K_1$  and  $K_2$  by multiple regression; 2) all three parameters ( $T_0$ ,  $K_1$ ,  $K_2$ ) of equation (4) were determined by multiple regression analysis.

## 3. Dependence of denaturation enthalpy on hydroxyproline content.

Regression analysis of the dependence of denaturation enthalpy on hydroxyproline concentration was undertaken using the results obtained by us earlier for fibril-forming collagen [16] (Table 2). Combining these data more than doubled the range in hydroxyproline concentration but did not change the general downward-curving form of this dependence (Fig.2). Regression equations were as follows. from the equation (9):

$$D_d H_r = 3.35 + 0.048 N_{Hyp} - 1.8910^{-4} N_{Hyp}^2. \quad (9)$$

The decrease of denaturation enthalpy after passing the maximum value occurs because the denaturation enthalpy of (Gly-Pro-Hyp) triplets is less than that of

(Gly-X-Hyp) triplets [17], and after the maximum the concentration of (Gly-Pro-Hyp) triplets increases with hydroxyproline, while (Gly-X-Hyp) concentration declines.

There is a possibility to check how (Gly-X-Hyp) and (Gly-Pro-Pro) triplets behave with the increase of hydroxyproline content in phylogenesis. Fig 4 presents the dependence of the probable number of  $N_{(GPO)}$  on the hydroxyproline content which can be expressed by the following relation:

$$N_{\text{Gly-Pro-Hyp}} = 7.92 + 0.007N_{\text{Hyp}} + 0.0036N_{\text{Hyp}}^2, \quad (10)$$

while the dependence of  $N_{\text{Gly-X-Hyp}}$  on the hydroxyproline content is the following:

$$N_{\text{Gly-X-Hyp}} = -10.02 + 1.08N_{\text{Hyp}} - 0.0036N_{\text{Hyp}}^2. \quad (11)$$

Empirical relations indicate quite obviously that the competing effect between these triplets should occur with the increase of hydroxyproline content.

We can calculate the concentrations of hydroxyproline at which the enthalpy of denaturation is at a maximum by equating the differential coefficient to zero. Thus, from Eq. (9), the maximum enthalpy occurs at  $N_{\text{Hyp}}=158$  residues /1000 residues:

$$[(d(\Delta H_r)/dN_{\text{Hyp}})]_{\text{max}} = |0.041 - 2.6 \cdot 10^{-4}N_{\text{Hyp}}| = 0;$$

$$N_{\text{Hyp}} = 0.041/2.6 \cdot 10^{-4} = 158 \quad (12)$$

and from equation (11), the maximum concentration of Gly-X-Hyp triplets occurs at  $N_{\text{Hyp}}=150$  residues /1000 residues:

$$[dN_{\text{Gly-X-Hyp}}/dN_{\text{Hyp}}]_{\text{max}} = |1.08 - 6.58 \cdot 10^{-3}N_{\text{Hyp}}| = 0;$$

$$N_{\text{Hyp}} = 1.08/7.2 \cdot 10^{-3} = 150. \quad (13)$$

We note that the maximum denaturation temperature occurred at the same hydroxyproline concentration ( $N_{\text{Hyp}}=155$  residues/1000 residues, see above).

## DISCUSSION

This analysis has shown that the concentrations of 4-hydroxyproline residues at which the denaturation temperature ( $T_d$ ), the denaturation enthalpy ( $\Delta H_r$ ) and the concentration of triplets (Gly-X-Hyp) are at a maximum Table 2.

Calorimetric data of  $\Delta H_r$ , and  $T_d$  of the collagen different origins [16].

Collagen	$N_{\text{Hyp}}/1000$ res.	$T_d$ °C	$\Delta H_r(25)$ kJ/mol-res.	$\Delta H_r(T_d)$ kJ/mol-res.
1. Antarctic icefish ( <i>Tr. Eulephidotus</i> )	45	6	5.08	4.85
2. Cod skin ( <i>Gadus sp.</i> )	53	15	5.49	5.36
3. Spiny dogfish ( <i>Squalus acanthias</i> )	59	16.3	5.47	5.39
4. Pike skin ( <i>Exos sp.</i> )	70	27	5.8	5.91
5. Carp swim bladder ( <i>Cyprinus carpio</i> )	81	32	5.98	6.15
6. Albino rat skin	93	37	6.21	6.48

and approximately equal: 155, 158, 150 residues per 1000 residues respectively. This is not accidental. As the imino acid concentration increases from cold-blooded to warm-blooded animals the number of (Gly-Pro-Hyp) triplets increases parabolically and the available stock of X positions that are not Pro declines. This decreases the number of water bridges associated with each hydroxyproline, thus affecting the enthalpy and temperature of denaturation.

In a previous paper [18], we estimated that the stabilizing influence of (Gly-X-Hyp) triplets was 8 times more than (Gly-X-Y) triplets and explained this, as here, on the basis of water-bridged structure of collagen, offered by Ramachandran et al. [4]. However, by utilizing calorimetric data for the denaturation enthalpy of collagens of various origin, the analysis executed in the present work reveals not only the temperature stabilizing superiority of (Gly-X-Hyp) triplets over (Gly-Pro-Hyp), but also provides thermodynamic support for the water-bridged collagen structure.

From the dependence of denaturation enthalpy on hydroxyproline content (Fig.3, Eq. (9)) the enthalpy contribution of triplets (Gly-X-Hyp) to total denaturation enthalpy of collagen molecule can be estimated. At the standard condition (25°C) it is 21.7 kJ/mol-residues and almost two times more than enthalpy hydroxylation of proline in the third position of triplets – 12.5 kJ/mol-residues [19], while at the denaturation temperature it is 31.5 kJ/mol-residues (Table 2). It is a very big value and means that the water-bridges in triplets (Gly-X-Hyp) participate in change of collagen thermostability too.

The main conclusions of the present analysis in comparison with the previous similar analysis [8,9] are: 1) not all hydroxyproline residues participate in an increase in thermostability; 2) increased thermostability is of mainly enthalpic rather than entropic nature, as proved earlier [7], and the phylogenetic change of collagen thermostability is achieved by changing the concentration of water-bridges connected with 4-hydroxyproline in (Gly-X-Hyp) triplets.

ბიოფიზიკა

## კოლაგენის სამმაგი სპირალის თერმოსტაბილობის ევოლუციური ცვლილების მოლეკულური მექანიზმი

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(წარმოდგენილია აკადემიკოს მ. ზაალიშვილის მიერ)

ზერხემლიანთა ფიბრილარულ კოლაგენებზე ჩატარებულმა ანალიზმა გვიჩვენა, რომ თერმოსტაბილობის დამოკიდებულება 4-ოქსიპროლინის ნაშთების რაოდენობაზე საშუალებას გვაძლევს განვსაზღვროთ (Gly-X-Hyp) და (Gly-Pro-Hyp) ტრიპლეტების წვლილი კოლაგენის თერმოსტაბილობის ცვლილებაში. პირველ მიახლოებაში დენატურაციის ტემპერატურის სიდიდე განიხილება როგორც (Gly-X-Hyp) და (Gly-Pro-Hyp) ტრიპლეტების ხაზოფანი ფუნქცია:

$$T_d = T_0 + K_1 N_{(GXO)} + K_2 N_{(GPO)},$$

სადაც კოეფიციენტები  $K_1$  და  $K_2$  განსაზღვრავენ ამ ტრიპლეტების ზემოქმედების ხარისხს. ანალიზმა გვიჩვენა, რომ (Gly-X-Hyp) ტრიპლეტები, რომლებიც შეიცავენ წყლის მოლეკულებს, იწვევენ თერმოსტაბილობის ზრდას, იმ დროს, როდესაც (Gly-Pro-Hyp) ტრიპლეტები განსაზღვრავენ დენატურაციის ტემპერატურის არაწრფივ დამოკიდებულებას. იგივე შედეგი მიიღება ენთალპიის ანალიზისას.

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