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Chapter 7

Unique Assembly Structure of Human Haptoglobin Phenotypes 1-1, 2-1, and 2-2 and a Predominant Hp 1 Allele Hypothesis

Mikael Larsson, Tsai-Mu Cheng,
Cheng-Yu Chen and Simon J. T. Mao

Additional information is available at the end of the chapter

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1. Introduction

Haptoglobin (Hp) is an acute phase protein present in the plasma of all mammals [1, 2]. One important function of Hp is its high binding affinity to hemoglobin (Hb) in forming the Hp-Hb complex that is metabolized through a receptor mediated process involving CD 163 of macrophages[3, 4]. This function is clinically relevant since Hb possesses a highly oxidative heme-group, producing reactive oxygen species when released from the red blood cells. On the contrary, Hp is a potent antioxidant which is stronger than the therapeutic agent probucol, which protects cells against oxidative stress [1, 5].

In humans there are two common alleles, *Hp 1* and *Hp 2*, corresponding to $\alpha 1\beta$ and $\alpha 2\beta$ polypeptide chains, respectively[3, 6]. All the phenotypes share the same β chain that is comprised of 245 amino-acid residues (Mw 40 kDa). As shown in Figure 1A, $\alpha 1$ contains 83 amino-acid residues (Mw 9 kDa) and possesses two free –SH groups. The one at the –COOH terminus Cys-72 always crosslinks with a β chain to form a basic $\alpha\beta$ unit or ($\alpha 1\beta$), and the other one at the NH2-terminus Cys-15 has to link with another ($\alpha 1\beta$) unit resulting in a Hp dimer ($\alpha 1\beta$)2 or Hp 1-1. In contrast, the $\alpha 2$ chain contains the same residues as $\alpha 1$ with an extra redundant copy of residues 12-70 (Figure 1A) giving a final 142 amino-acid residues (Mw 16. 5 kDa). It is "trivalent" with one extra free –SH group (Cys-15) that is able to interact with an additional $\alpha\beta$ unit. As such, one $\alpha 2\beta$ unit binds to either $\alpha 1\beta$ or $\alpha 2\beta$ to form large polymers [($\alpha 1\beta$)2-($\alpha 2\beta$)n in Hp 2-1 and ($\alpha 2\beta$)n in Hp 2-2] as shown in Figure 1B. Therefore, the different number of -SH sites produced from the two alleles lead to three phenotypes, each with a unique arrangement of polymers. For Hp 2-1, the ($\alpha 2\beta$) units form linear polymer chains, elongating

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until two (α1β) units bind to each side of (α2β)n so that (α1β)(α2β)n(α1β) polymers are formed. Notably, these polymers contain Hp 1-1 molecules, but not 2-2. For Hp 2-2 lacking of(α1β), the basic (α2β) units initially form linear polymers until the two endslink together to form a cyclic complex (α2β)n as illustrated in Figure 1B. These types of polymer structure have been confirmed by electron microscopic images [7].

Hp binds Hb with an extremely high affinity [2] and the latter possessing an endogenous peroxidase activity, it becomes a simple and popular routine method to identify the Hp phenotype using a peroxidase based colorimetric-substrate [8, 9]. As shown in Figure 1C, it is rather convenient to distinguish the phenotypes Hp 2-1 and 2-2 by observing the presence of (α1β)2 dimers and (α1β)2(α2β) trimers in Hp 2-1 and the presence of other higher order polymers in Hp 2-2. Interestingly, we have found that there are only few cyclic trimers among the Hp 2-2 polymers as compared to that of large intermediate polymers (Figure 1C). The variance in polymeric forms of Hp 2-1 and 2-2 and the mechanism by which Hp 2-1 possesses (α1β)2 with no cyclic (α2β)n have not been fully elucidated.

Clinically, the polymeric Hp phenotypes have been reported to be associated with the risk of kidney failure, diabetes, autoimmune, and cardiovascular diseases [6, 8, 10, 11].It is of interest to note that the plasma concentrations of Hp 1-1are found to be differentially higher than that of 2-1 and 2-2[8, 12]with values of 184 ±42, 153 ±55 or 93 ±54 mg/dL for Hp 1-1, 2-1 or 2-2, respectively [8].

The purpose of this study was to identify the possible number of polymers in each isolated Hp phenotypes 2-1 and 2-2 and to provide a theory for the Hp polymer assembly from our experimental data. We hypothesized that steric hindrance acts as a limiting factor on the formation of Hp 2-2 trimers. Finally, we addressed the differentially higher plasma levels of Hp 1-1 than 2-1 and 2-2 in normal subjects based on mRNA levels. A predominant gene activity of *Hp 1* greater than *Hp 2* was proposed.

2. Number of polymers in Hp phenotypes

To determine the number of polymeric forms of Hp 1-1, 2-1, and 2-2, plasma Hp isolated from a monoclonal antibody affinity-column [13]was first analyzed on a SDS-PAGE. A typical example showing the polymer numbers of Hp 2-2 is depicted in Figure 3. Western blot analysis confirms that each band actually corresponds to each size of the Hp polymer using a monoclonal antibody specific to the Hp α-chain (Figure 3B). Similar to that previously described [14, 15], the resolution using SDS-PAGE was not quite satisfactory. We then utilized a native-PAGE that gave a better resolution of higher polymers. Figure 3 shows that Hp 1-1 possesses a single homogenous form or (αβ)2 as expected, while Hp 2-1 or 2-2 possesses (αβ)2, trimer, tetramer, pentamer, and other polymers consistent to those depicted in Figure 2. The visible number of Hp 2-1 polymers is approximately up to 9 or (αβ)10, starting with (α1β)2. Remarkably interesting, there are as many as 18 polymers or (αβ)20 seen in Hp 2-2, which have not been previously identified in terms of the polymeric number.

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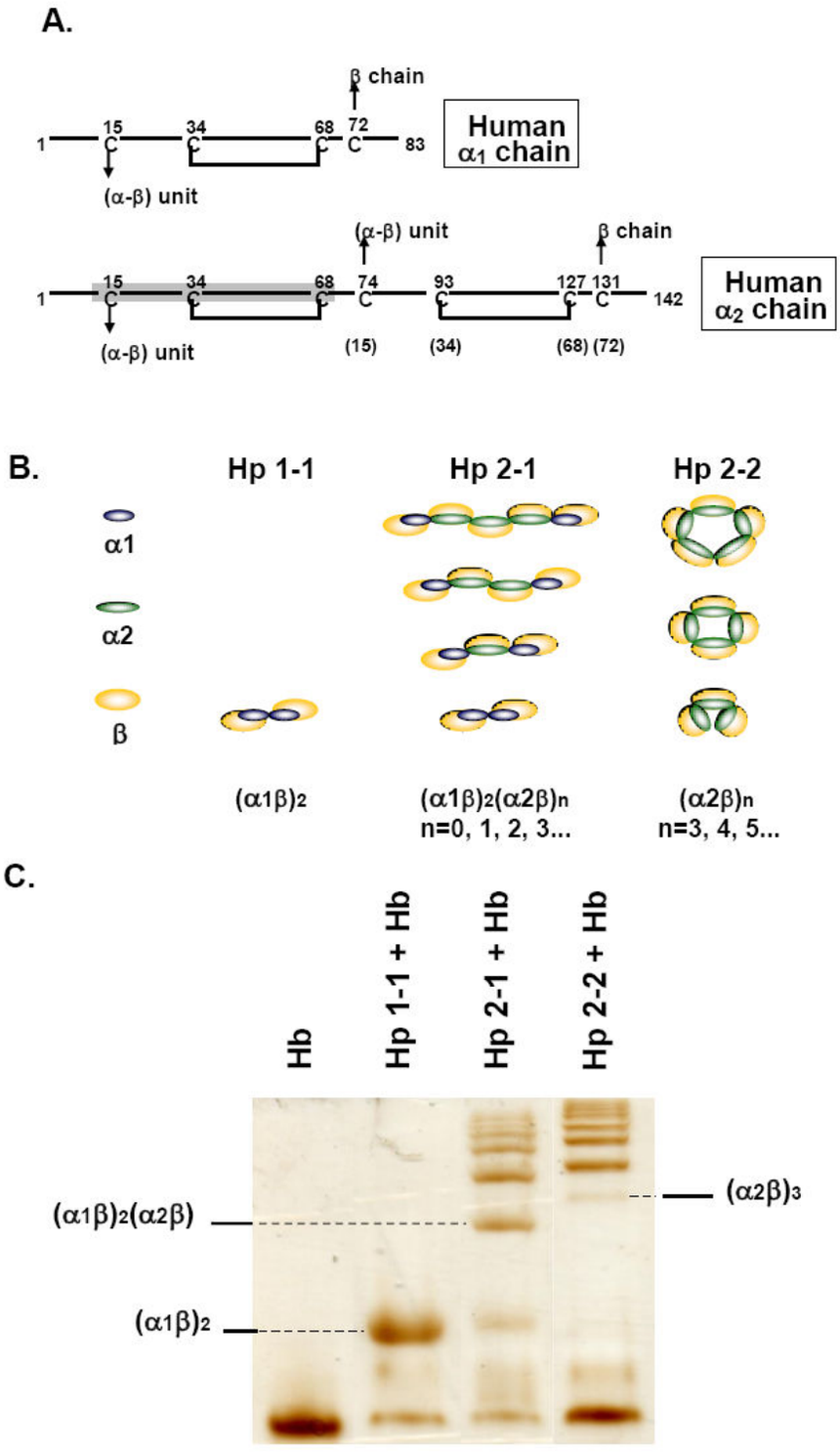


Figure 1. Polymeric structures of Hp 1-1, 2-1 and 2-2. Haptoglobin polymers are made up of (αβ) units with different number of –thiol groups in α1β and α2β. A) Schematic view of α1 and α2 chains encoded by the *Hp 1* and *Hp 2* alleles, respectively. The –COOH terminal Cys-72 of α1 always links to a β chain forming αβ basic unit. Whereas α2 contains a tandem repeat of residues 12-70 with Cys-15 and -74 linking to other αβ units, making the α2"trivalent". B) Illustrative view of the arrangement of (αβ) unit in each Hp phenotype, where n represents the repeat unit. C) 7% Native-PAGE of Hp-hemoglobin complexes showing the characteristics of polymeric pattern of each phenotype. Such complexes are used for Hp phenotyping. Of note, the amount of cyclic Hp 2-2 trimer or (α2β)3 is relatively limited.

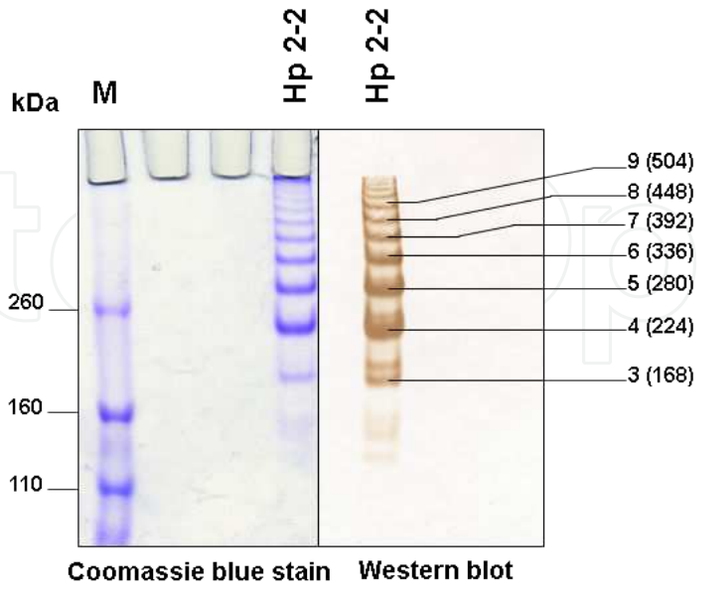


Figure 2. SDS–PAGE of isolated human Hp 2-2. Molecular patterns of polymeric Hp 2-2 using 4% non-reducing SDS—PAGE, showing the heterogeneous nature (left). The identity of each polymeric band is further confirmed by Western blot analysis using a α chain specific mAb (W1) prepared against Human Hp(right).

3. Decreasing concentration of Hp polymers determine the higher polymer numbers of Hp 2-2 than that of Hp 2-1

In addition to the number of Hp polymers, it is of interest to note that the concentration of each polymer is almost conversely correlated to the number of repeated ($\alpha\beta$) units. To substantiate the above observation, the relative intensity of each polymer within the Hp 2-1 or 2-2 was determined using an image analysis. Figure 4 demonstrates that the concentration of each polymer (except from Hp 2-1 dimer and Hp 2-2 trimer, discussed below) gradually decreases when the number of repeated units increases. The regression of arbitrary polymer concentration is found to be exponentially dependent on the number of repeated ($\alpha\beta$) units (starting from the trimer for Hp 2-1 and tetramer for Hp 2-2) leading to anequation for Hp 2-1 as:

$$\left[\mathrm{Pn}\right] = 6.4929 * e^{-0.5286n} \tag{1}$$

Where, n (≥ 3) represents the number of repeated units. [Pn] is the arbitrary protein concentration at the denoted number of repeated ($\alpha\beta$) units (n). The concentration of large polymers eventually attenuates to zero as the polymer number increases. Similarly, the equation for Hp 2-2 is:

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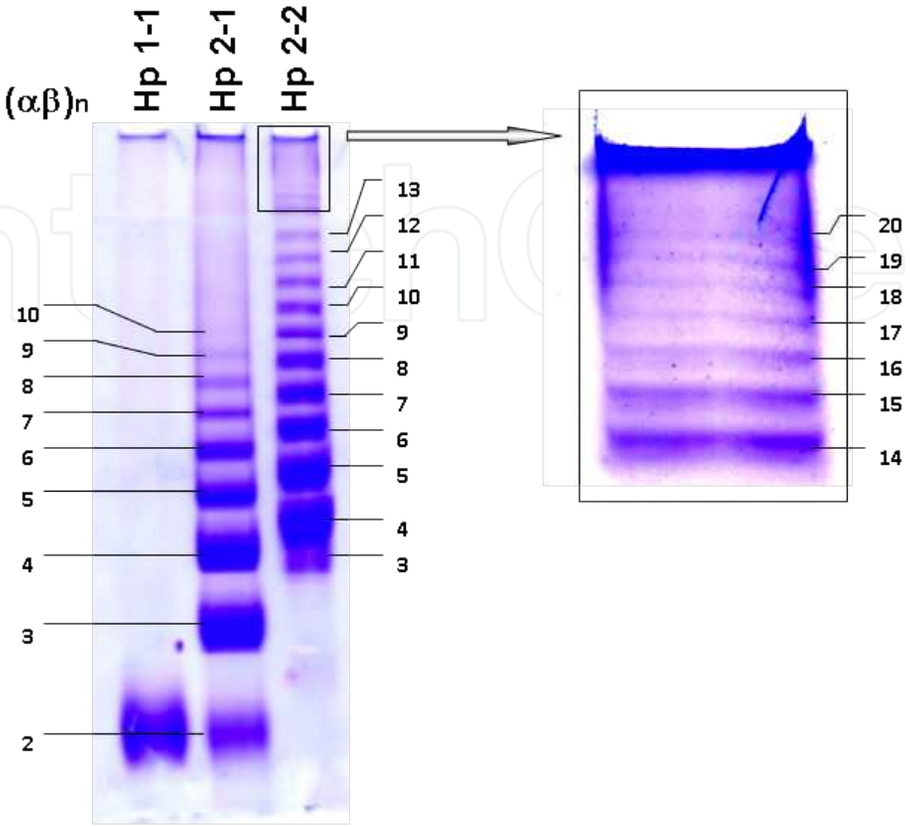


Figure 3. Native-PAGE of isolated human Hp 1-1, 2-1 and 2-2. For Hp 1-1 there is only a single homodimer or (α1β)2 observed, while polymers with as many as 10 and 20 repeated units can be seen in Hp 2-1 and Hp 2-2, respectively.

$$\left[\mathrm{Pn}\right] = 3.1783 * e^{-0.3319n} \tag{2}$$

Where $n \geq 4$

An exponential decrease is recognized as the solution to the differential equation:

$$\frac{d[p]}{dn} = -k\left[p\right] \tag{3}$$

This means that the relationship (or ratio) between the concentration of polymers at number n+1 and n is constant. For Hp 2-1, the quotient derived form Eq. (1) is:

$$6.4929 * e^{-0.5286\,(n+1)} / \; 6.4929 * e^{-0.5286\,n} = 0.59 \tag{4}$$

For Hp 2-2, the quotient from Eq. (2) is:

$$3.1783 * e^{-0.3319\ (n+1)} \ / \ 3.1783 * e^{-0.3319n} = 0.72 \quad (5)$$

Of notice that at a given protein concentration of Hp, the total number of detectable polymers for Hp 2-1 would be significantly less than that of 2-2. This is because the quotient in Eq. (4) is smaller, the protein concentration [Pn] approaches to zero faster (as n increases) when compared to Eq. (5).

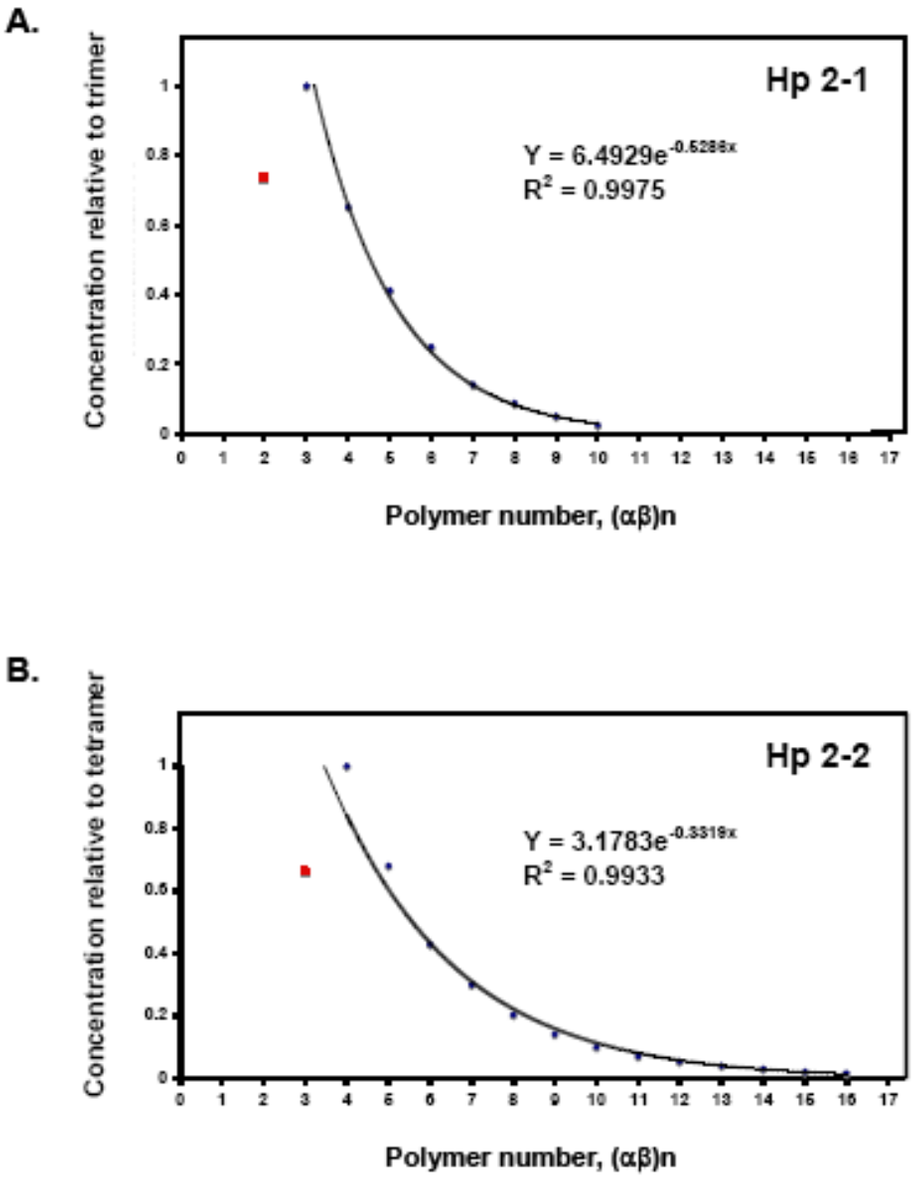


Figure 4. Plot of the polymer concentration as a function of (αβ)repeated number. A) Each polymer concentration of Hp 2-1 derived from Figure 3 is presented with the relative ratio to Hp trimer. The dimer is excluded from the exponential regression line since its concentration is beyond or below that proposed mathematical model (see text). B) Similarly, the trimer is excluded from the regression line as described above.

4. Possible kinetics for the assembly of Hp polymers

As shown in Figure 4 and Eq. (1) and (2), the concentration of the denoted polymer decreases exponentially with increasing polymer number. One of the attractive modes of elongation in polymer assembly (based on the exponential decrease in concentration) is that the polymer can be constructed by the addition of one ($\alpha\beta$)-unit at a time. This model assumes that reactions between already existing polymers are ignored. For example, a reaction such as: (α2β)n+1 and (α2β)n-1 to form (α2β)2n is not considered. However, if the reactions are multiples as that depicted in Figure 5, the possible assembly pathways of a given tetramer or hexamer would be complicated. Under the latter circumstance (Figure 5), once these multiple reactions take place it would generate a given polymer at different rates. We cannot rule out this possibility at the present time. The addition of one ($\alpha\beta$)-unit at a time is a simple model that gives rise to an exponential decrease with quotient remaining to be constant when applied to the number of polymers formed in each Hp phenotype. Despite the feasibility of the "one at a time" model, the overall rate of formation of polymers would be:

$$R_n > R_{n+1} \tag{6}$$

Where R_n is the formation rate of a denoted polymer with n repeated units, starting with n = 3 for Hp 2-1 and n = 4 for Hp 2-2 (Figure 4) (discussed below). It means that the smaller the polymers, the higher rate of assembly.

5. Proposed scheme for the formation of Hp 2-1 linear polymers

It has been well established that Hp 2-1 polymers, attributed by heterozygous *Hp 1* and *Hp 2*, are in a linear form (Figure 1). One essential question we attempted to address is why an Hp1-1 molecule can be seen without cyclic Hp 2-2 polymers in Hp 2-1 populations (Figures. 1 and 3). As depicted in Figure 6A, the gene responsible for the synthesis of α1β and α2β are from the *Hp 1* and *Hp 2* alleles, respectively. In theory, some α2β should be able to form 2-2 cyclic polymers. We speculated that the overall α1β-mRNA synthesized might be greater than the α2β-mRNA, which is in favor of the initial assembly of Hp 1-1 dimer (α1β)2. To test this hypothesis, we used the HepG2 cell line which by coincidence belongs to the Hp 2-1 genotype. We then determined the expression levels of the *Hp 1* and *Hp 2* alleles over time using RT-PCR, while utilizing LDL as an acute phase stimulant [8]. Figure 6B and C demonstrate that the expression of the *Hp 1* allele is significantly superior to *Hp 2* through all the induction times. In a previous study, we also reported that the synthesis rate of α1β was significantly faster than that of α2β after induction [8].

First, we proposed that the excessive (α1β) units naturally self-assemble into Hp 1-1 molecules. Second, because each (α2β)-unit contains two -SH open ends, (α2β) units must initially self-assemble into (α2β)n regardless of the presence of (α1β), where n ≥ 2. As shown in Figure 7 using a pentamer as an example, the reaction of cyclization of (α2β)n may take a long time in

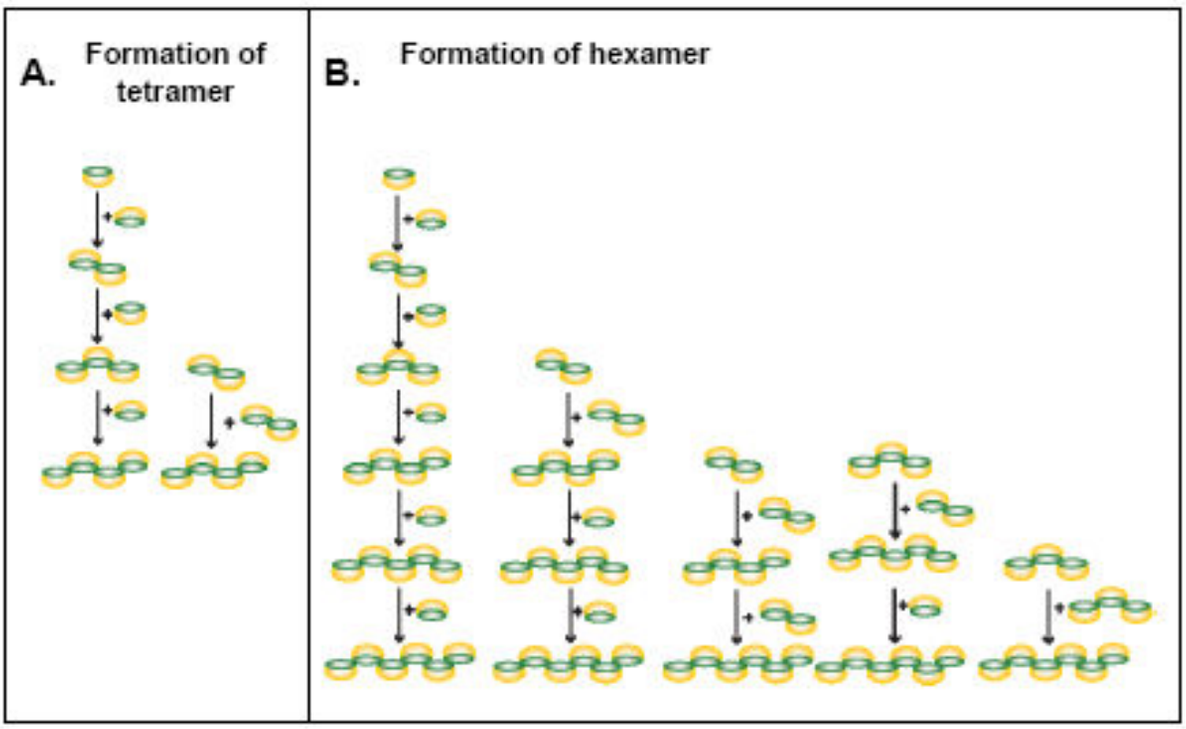


Figure 5. Schematic view of large and small polymers assembled through multiple reaction pathways. A) An example using tetramer as a model for two pathway assembling, one is by adding one unit at a time and the other is via a reaction between the two dimers. B) An example using hexamer as a model to illustrate there are five possible pathways, including by adding one unit at a time and by reactions between already formed polymers.

the process of polymer refolding. The rate of cyclization in theory is slower than the formation of linear polymers. In the presence of excess (α1β) units, these (α2β)5 polymers in linear form could be terminated by the addition of (α1β) at both ends. The resulting product is, therefore, in a linear form.

Furthermore, Figure 8 shows that one (α1β) and one (α2β) can also form (α1β)-(α2β)-, but is terminated with an addition of a (α1β) which gives rise to the smallest heterogeneous linear polymer (α1β)-(α2β)-(α1β). However, if the next addition is a (α2β), then further extension by coupling an (α1β)- or (α2β)-unit is possible. Thus, the next linear polymer is a tetramer or (α1β)-(α2β)-(α2β)-(α1β), otherwise the elongation continues until the addition of a (α1β). If the portion of open-end polymers (α1β) (α2β)n- that adds (α2β) or (α1β) is independent of the number of repeated ($\alpha\beta$)-units (n) within the polymers, then the relationship between the rate of addition of (α1β) and (α2β) does not change. Thus, the concentration of polymers depending on size would follow an approximately exponential decrease since a constant portion of an open end polymers adds (α1β) and a constant portion adds (α2β) (Figure 8). However, since there are two different pathways leading to polymers with only one open end (Figure 7), the decrease is truly exponential if the (α1β) are added in same positions for the both pathways.

Explaining the low abundance of dimers or (α1β)2 in Hp 2-1of Figure 3 is somewhat difficult. One possible explanation is that formation of the (α1β)2 is terminated and limited by the presence of (α2β)-subunits. If this was the case, the reactivity of the (α2β) could be higher than

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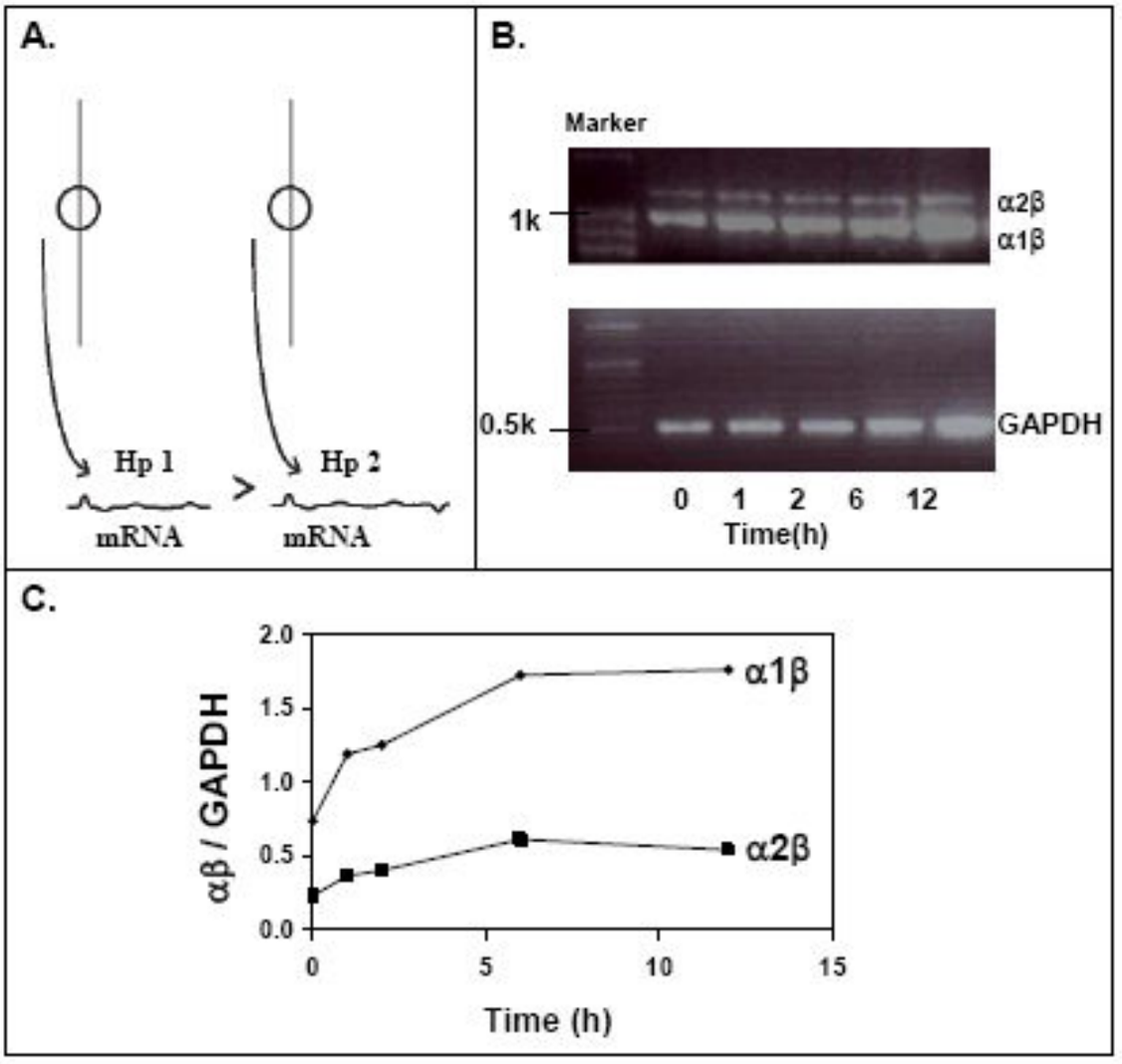


Figure 6. Schematic view of the molecular expression of α1β and α2β unit and its level induced by LDL. A) *Hp 1* and *Hp 2* alleles are responsible for making the mRNA of α1β and α2β polypeptides, respectively. B) RT-PCR showing the overall synthesis of α1β is greater than α2β m-RNA over time, while the house keeping gene GAPDH was used as a control. C) The intensity of the α1β band is higher than that of α2β at all induction times as determined using an image analysis.

that of (α1β) in terms of the binding to the other ($\alpha\beta$)-units. Nevertheless, it is a fact that the concentration of Hp 1-1 determined in Hp 2-1 polymers is not fit into the exponential decrement curve shown in Figure 4A.

6. Proposed scheme for the formation of Hp 2-2 cyclic polymers

As shown in Figure 9, each basic (α2β) unit initially forms linear polymers with both ends having one thiol group open for further subunit extension. The elongation terminated until the free ends bind together to form a cyclic Hp 2-2. According to Eq. (5), the quotient between

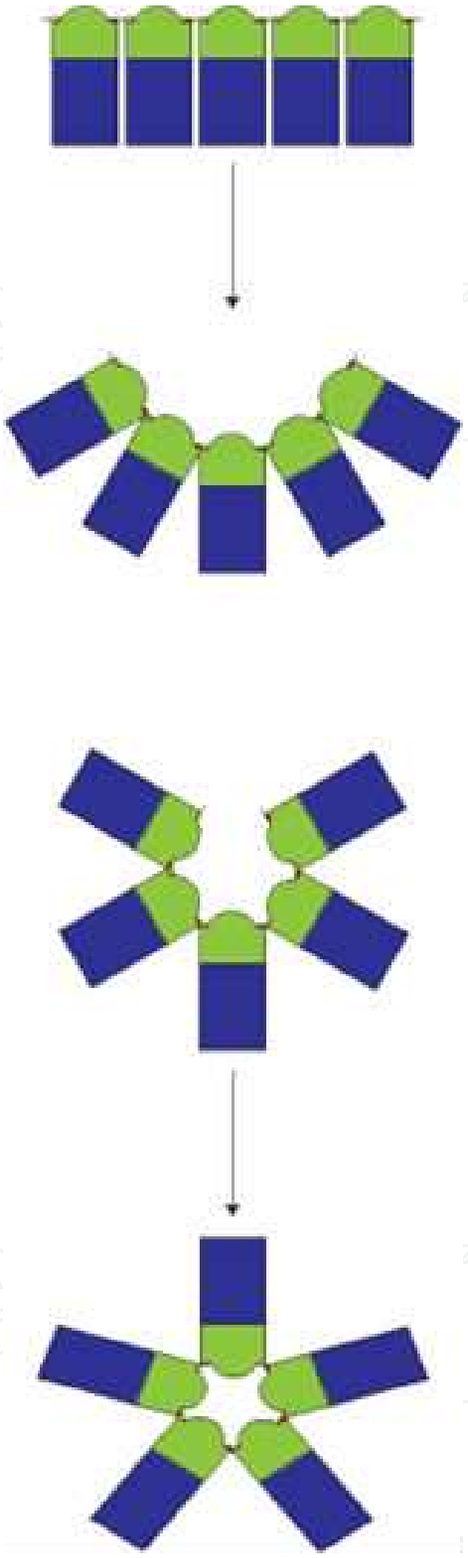


Figure 7. Model of cyclization of Hp polymers. Regardless of the pathways involved in the formation of (α2β)n, a given pentamer or (α2β)5 requires a correct conformation to be cyclized. The rate of the process is therefore slower than uncyclized form. The uncylized (α2β)n could be initially present in the polymer populations during the assembly of Hp 2-1 molecules. However in the presence of excess of basic (α1β) units, the remaining uncyclized forms are terminated by coupling a (α1β). If (α2β) units are in excess, the cyclization of Hp 2-2 polymers should be allowed in theory.

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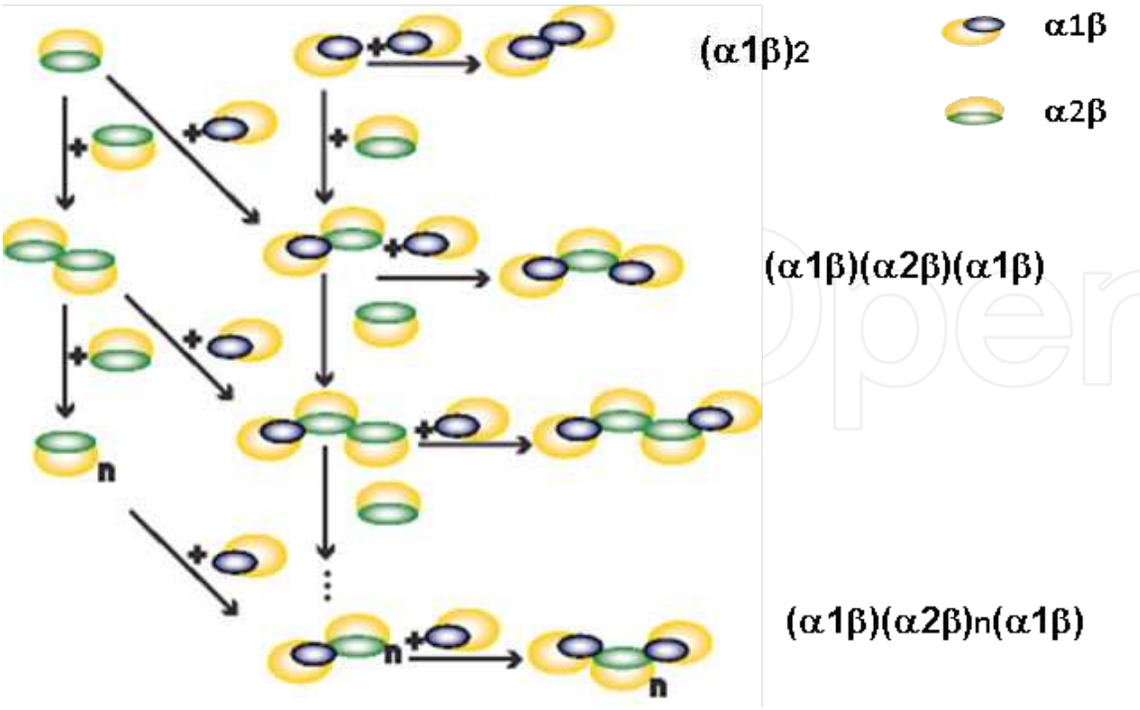


Figure 8. Proposed model for the formation of Hp 2-1 linear polymers. Nucleation occurs through the reaction between the two αβ units with the possible products: a simple (α1β)2 dimer is initially formed without further extension because of the saturation of free -thiol groups. The next linear trimer is formed with the addition of two (α1β) units to one (α2β). Notably, either (α1β) or (α2β) may subsequently add to one (α2β) unit until both ends are bound with (α1β).

polymers of order n+1 and n is 0. 72. It means that 72% of the polymers with free –SH would link another ($\alpha 2\beta$) to form the next higher order polymer. It equals to:

$$\text{Rate of addition } \alpha 2\beta \ / \ (\text{Rate addition of } \alpha 2\beta \ + \ \text{Rate of cyclic formation}) \ = 0.72. \tag{7}$$

Which equals to:

$$\text{Rate of cyclic formation } = \ 0.39 \ * \text{rate of addition of } \alpha 2\beta. \tag{8}$$

It is seen from Eqs. (7) and (8) that the formation of a cyclic polymer is slow as compared to the addition of a ($\alpha 2\beta$)unit. In other words the ratio between the next order of a polymer and a given polymer remains constant or 72% (Figure 3). This is thought to be the reason for the large polymer numbers seen in the Hp 2-2 phenotype. The slow rate of cyclic formation further explains why there are no cyclic ($\alpha 2\beta$)n polymers found in the Hp 2-1 population. However, if the overall synthesis of ($\alpha 2\beta$) molecules was in excess in the individuals possessing both *Hp 1* and *Hp* heterozygote (Hp 2-1 phenotype), we would see the cyclic polymers regardless of the slow rate involved in the cyclization. Nevertheless, the rate of cyclization derived here is consistent to that we proposed in Figure 7.

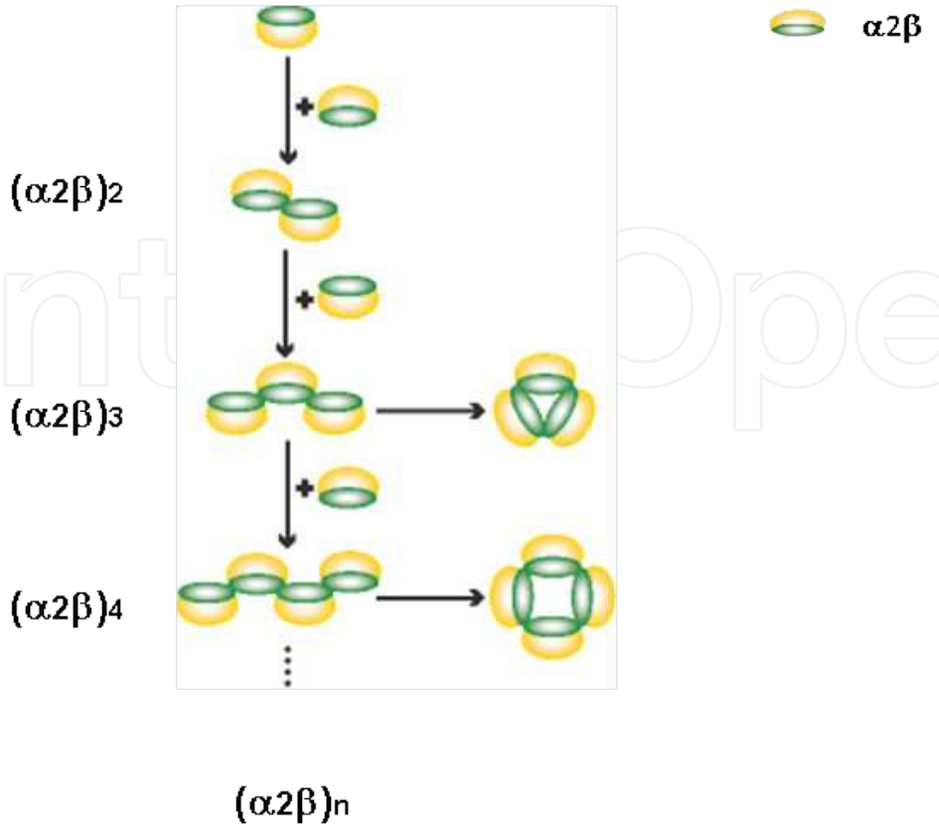


Figure 9. Hypothetical model for the formation of cyclic polymers. Nucleation occurs through the reaction between two (α2β) in creating a first linear (α2β)2. Formation of a stacked dimer is not possible due to the steric hindrance between the two -thiol groups of each subunit (depicted in Figure 10). As such it initially forms a linear trimer or (α2β)3 prior to the cyclization. This linear trimer can then either be elongated by the addition of other (α2β)n, or otherwise be terminated by a subsequent cyclization.

7. Steric hindrance could explain the low abundance of trimer in Hp 2-2 phenotype

A fascinating phenomena is that the concentration of Hp 2-2 trimers or (α2β)3 seen in Hp-Hb complex is extremely low in human plasma of all the Hp 2-2 subjects that we have investigated without exception (Figure 1C). Its concentration does not fit the mathematical model (Figure 3 and eq. 2). As depicted in Figure 10, we hypothesized that there is a steric hindrance between the two free thiol groups of a (α2β) unit. First, under this condition the hindrance totally abolishes the formation of a basic dimer (α2β)2. Second, steric hindrance prevents the cross-linking from forming a trimer to some extent due to limited space in the central space. This may account for the low abundance of trimers in Hp 2-2 polymers. Third, as the central space increases, the hindrance does not substantially affect the formation of a tetramer, a pentamer, or larger polymers. Recently, we also demostrated an unique tetrameric structure of deer plasma Hp which cna explain an evolutionary advantage in the Hp 2-2 phenotype with homogeneous structure[16].

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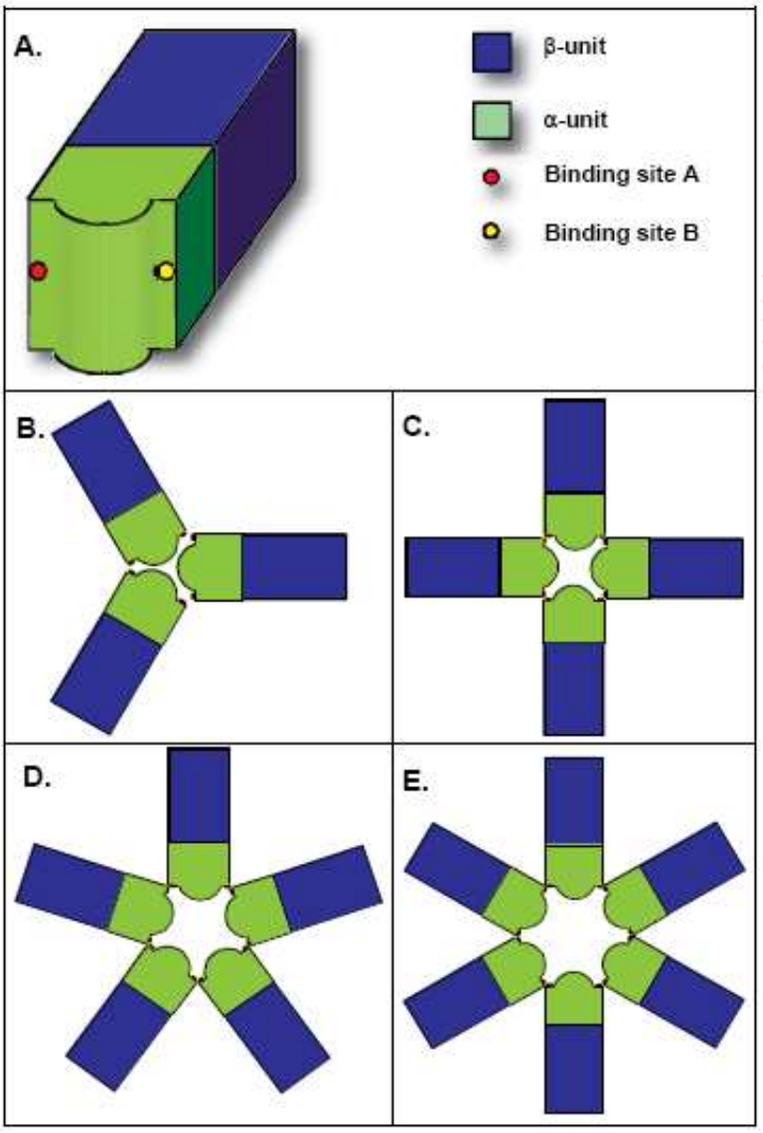


Figure 10. Proposed model of the assembling of Hp 2-2 polymers with limited trimer molecules. A) The two -thiol groups linking the Hp subunits into polymers are located at a plane where they are separated by a steric hindrance. Under this condition the hindrance prevents the formation of a basic dimer (α2β)2. B) The trimer is able to form to some extent, but is limited by the hindrance that accounts for its low abundance. C-E) The polymers of order four and higher are assembled without any steric hindrance as the central space getting wider.

8. Plasma Hp 1-1 levels are differentially greater than Hp 2-1 and 2-2

It has been known that plasma levels of Hp 1-1 are dramatically higher than 2-1 and 2-2 in normal human subjects. Order of the levels is Hp 1-1 > Hp 2-1 > Hp 2-2 with plasma concentrations of about 180, 150 and 90 mg/dL, respectively [8]. The mechanism involved in such discrepancy, however, has not been explored. As shown in Figure 6A, there are two alleles *Hp 1* and *Hp 2* responsible for the specific synthesis of the (α1β) and (α2β) subunits, respectively. Based on the RT-PCR analysis of allele expression using a HepG2 cell line containing both alleles, it appears that the amount of mRNA produced by the *Hp 1* allele is significantly greater

than that of *Hp 2* over time (Figure 6B-C). Thus, it explains why the plasma concentration of Hp in subjects with *Hp 1* is markedly higher than that with *Hp 2-1*(heterozygote) or *Hp 2*(homozygote). Although the reason for low expression of the *Hp 2* allele remains unclear, we proposed that the gene activity of *Hp 1* is superior to *Hp 2*.

9. Clinical significance of Hp phenotypes

The Hp is an acute-phase protein in response to infection and inflammation. It is also one of the most abundant serum proteins with high potency of anti inflammation and antioxidant activities [17, 18];therefore render its availability for maintaining homeostasis. Anyaberrancein expression levels or subfraction composition of Hp may possibly be used to establish valuable diagnostic or prognostic indicator in various diseases. Human Hp polymorphism is not only determined by unique genetic duplication, but also affected by complex assembly processes.

Reports regarding the relationship between the plasma levels and diseases remain rare owing to the difficulty and complexicity in precised determination of Hp levels in different individuals with different phenotypes. We have shown for the first time that immunoassay (such as ELISA) for Hp measurement has to use Hp phenotype-matched standard for one individual with one specific phenotype (ie., one Hp 1-1 subject needs Hp 1-1 protein as a standard used for calibration) due to the different biochemical structure and immunochemical properties among the Hp phenotypes. Following this concept, we have established an accurate ELISA test for measurement of Hp levels [8]. Clinically, we discovered that the patients with normal acute-phase response in Hp elevation in sepsis had fewer events of multiple-organ dysfunction and lower mortality rate. The Hp2-2 phenotype is associated with failure to increase plasma Hp levels in the acute stage of sepsis (unpublished data). In another study, we showed that one short-term jogging and explosive run are able to induce a substantial elevation of Hp in peripheral blood. In mice, Hp levels are elevated significantly and concomitantly with the increase in neutrophils over the circulation following a 2-week exercise [19]. This finding not only suggests that acute net increase in Hp levels may be directly derived from the neutrophils but also indicates that Hp could be a biomarker for the neutrophil functional activity.

It is well known thatcardiovascular eventsin diabetic patients are closely linked to the Hp2-2 phenotype [20]. Recently, accumulating evidence showed that Hp elevation implies various biologic meaning. Notably, elevated Hp concentrations in cord blood of newborns have been identified as a biomarker to predict the occurrence of early-onset neonatal sepsis [21-23]. In patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and multiple sclerosis,Hp levels in cerebral spinal fluid are high [24]. Serum Hp is also a useful predictive biomarker for steroid therapy efficacy in the treatment of idiopathic nephrotic syndrome[25]. Plasma Hp concentrations are elevated in patients with abdominal aortic aneurysm, particularly those with the Hp 2-2 phenotype [26].

10. Conclusions

Regardless of the hypothetical model, the discovery of an exponential decrease in concentration between ($\alpha\beta$)n and ($\alpha\beta$)n+1 is of remarkable interest. It provides insight into the role of Hp polymer size involved in the clinical outcomes and physiological functions. The maximal number of repeated ($\alpha\beta$) units we reported here is as many as 10 for Hp 2-1 and 20 for 2-2. Should the concentration not follow an exponential decrement, the maximal number of polymers assembled could have been much larger. We suggest a simple kinetics model with the excessive synthesis of (α1β) units that can explain the lack of cyclic polymers in the Hp 2-1 individuals. We also proposed that the allele activity of *Hp 1* is superior to *Hp 2*, which accounts for the differentially greater Hp concentrations in Hp 1-1 human subjects than in Hp 2-1 and 2-2. Finally,we speculated that Hp polymorphism from genetic sequence and protein assembly may reflect the response to inflammation. The application of Hp in clinical medicine awaits further investigations.

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Author details

Mikael Larsson[1,2], Tsai-Mu Cheng[1,3], Cheng-Yu Chen[4,5,6] and Simon J. T. Mao[1]

*Address all correspondence to: doctormao888@gmail.com

1 Research Institute of Biochemical Engineering, Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC

2 Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

3 Taipei Medical University, Taipei, Taiwan, ROC

4 Department of Internal Medicine, Nation Yang-Ming University Hospital, Ilan, Taiwan, ROC

5 Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, ROC

6 Cardinal Tien College of Healthcare and Management, Taipei. Taiwan, ROC

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