Amplification of Antioxidant Activity of Haptoglobin(2-2)–Hemoglobin at Pathologic Temperature and Presence of Antibiotics

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Abstract It is clear that Haptoglobin binds to Hemoglobin strongly and irreversibly. This binding, protects body tissues against heme-mediated oxidative tissue damages via peroxidase activity of Haptoglobin–Hemoglobin complex. Peroxidase activity of Haptoglobin(2-2)–Hemoglobin complex was determined via measurement of following increase in absorption of produced tetraguaiacol as the second substrate of Haptoglobin–Hemoglobin complex by UV–Vis spectrophotometer at 470 nm and 42°C. The results are showing that peroxidase activity of Haptoglobin(2-2)–Hemoglobin complex is modulated by homotropic effect of hydrogen peroxide as the allosteric substrate. On the other hand, antioxidant activity of Haptoglobin (2-2)–Hemoglobin is increased via heterotropic effect of two antibiotics (especially ampicillin) on the peroxidase activity of the complex. The condition of pathologic temperature along with the administration of ampicillin and/or coamoxiclav is in favor of amplification in antioxidant activity of Haptoglobin(2-2)–Hemoglobin and combating against free radicals in individuals with Hp2-2 phenotype. Therefore, oxidative stress effects have been diminished in the population with this phenotype.

Keywords Haptoglobin · Hemoglobin · Antioxidant · Pathologic · Antibiotics

Introduction

Haptoglobin (Hp) is a unique acute phase protein that primarily scavenges the released hemoglobin (Hb) into the circulation by hemolysis or normal red blood cell turnover. Assessment of different Hp genotypes in different geographical areas of the world shows that there is no uniformity in distribution of different genotypes of this protein. In the northwestern parts of Europe, Hp1-1 genotype is carried by approximately 16% of individuals, Hp2-2 by 36% and Hp2-1 by 48% of the people (corresponding to frequency of alleles which is 0.4 for Hp1 and 0.6 for Hp2). The lowest frequency of Hp1 allele is reported from South-East Asia which is equal to 0.1 and the highest is in South America which is 0.8 [1]. Some recent studies have proposed possible associations between specific Hp genotypes/phenotypes and some particular disorders such as cardiovascular diseases, autoimmune disorders, psychiatric abnormalities and some of the cancers. It has been shown that Hp2 allele is associated with a higher levels of lipid peroxidation products than Hp1 allele. Also Hp2-2 phenotype has been found as a risk factor in many inflammatory diseases [1].

It has been demonstrated that ‘Free’ hemoglobin (Hb) which is released from red blood cells or red blood cell precursors into the plasma during the physiological and pathological hemolysis is as a highly toxic substance for human body. Free radicals such as $O_2^{\bullet-}$ and $\bullet OH$ are extremely reactive molecules that are able to cause cell damages by peroxidation of membrane lipids, proteins and nucleic acids [1]. Free Hb promotes accumulation of hydroxyl radicals ($\bullet OH$) by the heme iron which is able to

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react with endogenous hydrogen peroxide via Fenton and Haber–Weiss reaction to produce free radicals. These free radicals may cause severe oxidative cell damages [2, 3]. Furthermore the auto-oxidation of oxy Hb may produce superoxide ions. Hemoglobin shows a significant peroxidase activity in presence of endogenous oxidants such as H_2O_2 and either ascorbate as the second substrate [4]. Haptoglobin (Hp) is a serum acute phase protein which performs a soluble complex with Hb. The binding between Hp and Hb is very strong, stable and irreversible [1, 5, 6].

It has been reported that peroxidase activity of free hemoglobin is very low and so binding of haptoglobin to hemoglobin cause a considerable increase peroxidase activity of hemoglobin. Although Binding site of haptoglobin on hemoglobin is located on the globin moiety of the protein but the increased peroxidase activity of hemoglobin is related to the configuration of the heme group [7].

When Hb is released from red blood cells into plasma, it rapidly dissociates into $\alpha\beta$ -dimers which bind to Hp in a 1:1 stoichiometry, i.e. one Hb dimer per Hp ($\alpha\beta$)-unit [7, 8]. It is reported that both α - and β -chains of Hb are participated in Hp binding [9–11]. β globin chain of human Hb contains two specific binding sites for Hp which are at amino acid residues of β 11–25 and β 131–146, whereas Hb α globin chain has only one Hp-binding at α 121–127 [12].

Plasma concentration of Hp increases several folds in both local (vascular) and systemic (extravascular) in event of an inflammatory stimulus such as infection, injury or malignancy. It is hypothesized that neutrophils which are attracted to the sites of infection/injury release stored Hp to facilitate local clearance of ‘free’ Hb and so protect tissues from oxidative stress, following oxidative tissue damages and iron dependent bacterial growth [13].

Materials and Methods

Hydrogen Peroxide

A 0.5 M solution of hydrogen peroxide was prepared in phosphate buffer of 50 mM, pH 7.5. Peroxide solution was discarded after 30 min due to the necessity of fresh dilution preparation [14].

Guaiacol Reagent

A 0.03 M buffered solution of guaiacol was prepared as follows: 1.86 g of guaiacol and 50 ml of acetic were added to 400 ml of water. The volume was reached up to 500 ml with distilled water. The guaiacol reagent is stable for several weeks when it is stored in cold temperature [14].

Met-Hemoglobin Solution

Hemoglobin solution was prepared at a concentration of 10 mg/ml in 50 mM phosphate buffer, pH 7.5 phosphate buffer. An equal volume of 0.4 mM potassium ferricyanide was added to convert oxy-hemoglobin to met-hemoglobin completely. The resulted met-hemoglobin solution was then carefully diluted using the same buffer to 0.03 mM [14].

Haptoglobin2-2 Solution

A 0.03 mM solution of Hp2-2 was prepared in 50 mM phosphate buffer, pH 7.5 and the concentration of the solution was determined using the reported extinction coefficient of haptoglobin (ϵ mM 58.65 Hp2-2). Number of moles of Hp2-2 was based on its monomer properties because it has been assumed that each Hp2-2 monomer is capable of binding to a single hemoglobin molecule [5, 15].

Preparation of Hp(2-2)–Hb Complex

Hp(2-2)–Hb complex was prepared through combination of 50 ml of each of the above mentioned Hp2-2 and Hb solution and incubation at the room temperature for 30 min with gentle agitation to ensure that all Hp molecules were saturated with Hb.

Enzymatic Activity Assay

Peroxidase activity of Hp(2-2)–Hb complex was determined through the measurement of the following increase in

Fig. 1 Saturation curve for antioxidant activity of Hp(2-2)–Hb using hydrogen peroxide as substrate in phosphate buffer 50 Mm, pH 7.5 at 42°C. Each enzymatic assay was done using 0.03 M guaiacol as second substrate and following of A_{470} . Sigmoidal shape of the curvature shows non-michaelis behavior

Fig. 2 Non-Michaelis analysis of peroxidase activity of Hp(2-2)-Hb complex. **a** Eadie-Hofstee plot. The nonlinear shape and the curvature toward right show non-Michaelis homotropic allosteric effect. **b** Clearance plot. As it is observed the upward curvature confirms homotropic property. Maximum clearance (CL_{max}) and S_{max} have been determined graphically as shown. **c** Hill plot. It is seen the points of this graph lay on at least two consecutive linear parts. The slope of each line (m) is more than unit ($m > 1$). This observation not only

confirms positive cooperativity and homotropic effect but also demonstrates the behavior is changed with H_2O_2 concentrations so that the two sequential homotropic behaviors lead to increased activity. **d** Comparison between experimental and calculated saturation curve. Experimental initial velocities (continuous line) are directly from Fig. 1. Calculated initial velocities (dotted line) have been calculated using Hill coefficients (c) and Hill equation as explained in “Materials and Methods” section

Table 1 Enzymatic and allosteric parameters of antioxidant activity of Hp(2-2)-Hb complex in the presence of ampicillin and coamoxiclav

Complex	Drug	CL_{max} ($\times 10^6$)	S_{max} (M)	S_{50} (M)	m_1	m_2
Hp(2-2)-Hb	–	7.2	0.027	0.02	1.8	3.7
	Ampicillin	9.3	0.025	0.027	1.4	2.3
	Coamoxiclav	7.7	0.033	0.026	1.2	2.0

The values have been directly obtained from saturation curve (Figs. 1, 3) Clearance plots (Figs. 2b, 4b) and Hill plots (Figs. 2c, 4c, d) as it has been mentioned in “Materials and Methods” section

absorption of produced tetraguaiacol as the second substrate of Hb–Hp complex at 470 nm and 42°C by UV–Vis spectrophotometer (CICEL Model 1900) [16]. The assessed mixture was contained 64 nM Hp(2-2)–Hb complex, 0.03 M guaiacol and different concentrations of H₂O₂ in 50 mM phosphate buffer, pH 7.5 and the final volume was 1 ml. All components except Hp–Hb complex were added directly to a quartz cuvette and mixed via six times inverting. Then, 5 µl of Hp–Hb solution was added to the mixture and cuvette was inverted three times to mix the ingredients.

The exact time point at which Hb–Hp complex was added to the mixture was considered as the start time point of light absorption by the mixture at 470 nm. For each concentration of hydrogen peroxide the reaction was performed at least three separate times.

Determination of Enzymatic Parameters

Maximum initial velocities ($V_{\max}$) were obtained graphically using appropriate saturation curves. Amount of S_{50} (a concentration of H₂O₂ in which velocity of enzymatic activity is half of $V_{\max}$) was obtained from the H₂O₂ concentration which was related to the cross point of Hill plot to X-axis. The amount of ($CL_{\max}$) (maximum clearance) and $S_{\max}$ (a concentration of H₂O₂ in which $CL_{\max}$ occurs) were graphically obtained via the clearance plots.

Calculation of V-cal

Calculated initial velocity (V-cal) was obtained using Hill equation;

$$v = \frac{V_{\max} \times S^m}{S_{50}^m + S^m}$$

where $V_{\max}$ is maximum initial velocity, S_{50} is the substrate concentration which is corresponded to the half of $V_{\max}$, m is Hill coefficient (slope of Hill plot) and S is various substrate concentrations [17].

Results and Discussion

Haptoglobin has been known as the acute phase plasma glycoprotein [18, 19]. After more than 70 years laboratory research studies, haptoglobin is recognized as a multifunctional protein. It plays physiological roles in regulation of different processes including immune responses, angiogenesis, prostaglandin synthesis and reverse cholesterol transport [20, 21]. However, it still appears that the primary role of Hp is to bind to ‘free’ Hb and peroxidase activity. Therefore, it protects body tissues from heme-catalyzed

oxidative stress as well as facilitating Hb-uptake by macrophages [22].

Initially, determination of Hp values was based on enhancement of the peroxidase activity of Hb by Hp–Hb binding [5]. Afterwards, it was reported that peroxidase activity of free Hemoglobin is very low and binding of haptoglobin to hemoglobin cause a considerable increase in the peroxidase activity of hemoglobin. Although the increased peroxidase activity of hemoglobin is related to the configuration of the heme group, but the binding site of haptoglobin on hemoglobin is located on the globin moiety of the protein [5]. It has been denoted that the physiological importance of hydrogen peroxide removing is only in the acute condition. Due to the lack of kinetic studies in the previous literatures which are related to the peroxidase activity of Haptoglobin–Hemoglobin complex, the present study has investigated the kinetic properties of the complex enzymatic activities. The results indicate that the antioxidant activity of Hp(2-2)–Hb complex is analyzable using Michaelis–Menten model (due to the sigmoidal shape of its saturation curve, Fig. 1) and it requires providing an enzymatic model with the multiple sites to perform a cooperative binding to substrate. Eadie–Hofstee plot (Fig. 2a) confirms positive synergic results from right-side curvature of plot. In Fig. 2b, it has been shown that there is a well defined maximum for clearance of the substrate $CL_{\max}$. Although $CL_{\max}$ (like K_m) is determined under in vitro condition but it is an appropriate parameter to investigate the enzyme activity under in vivo condition. Highest concentration of hydrogen peroxide causes maximum clearance ($CL_{\max}$) which is named $S_{\max}$. Both

Fig. 3 Saturation curves for peroxidase activity of Hp(2-2)–Hb complex using hydrogen peroxide as substrate in phosphate buffer 50 mM, pH 7.5 at 42°C in the presence of therapeutic concentration of ampicillin and coamoxiclav. Saturation curve of Fig. 1 has been incorporated here, for comparison

Fig. 4 Non-Michaelis analysis of peroxidase activity of Hp(2-2)-Hb complex. **a** Eadie-Hofstee plot. The nonlinear shape and the curvature toward right show non-Michaelis homotropic allosteric effect. Eadie-Hofstee plot in the absence of drugs (Fig. 2a) has been incorporated here, for comparison. **b** Clearance plot. As it is observed the upward curvature confirms homotropic property. Maximum clearance ($CL_{\max}$) and $S_{\max}$ have been determined graphically as shown. Clearance plot in the absence of drugs (Fig. 2b) has been incorporated here, for comparison. **c** Hill plot in the presence of ampicillin. It is seen the points of this graph lay on at least two consecutive linear

parameters were obtained from Clearance plot (Fig. 2b). Because of the maximum complex auto-activation, $S_{\max}$ was observed in somewhat lower concentrations of hydrogen peroxide. Actually $CL_{\max}$ provides an estimation of the highest clearance ability of substrate before saturation of the enzyme active sites. Therefore, if this hypothesis that “in vivo activation of enzymes occurs via endogenous activators” is accepted, $CL_{\max}$ might be an appropriate

parts. The slope of each line (m) is more than unit ($m > 1$). This observation not only confirms positive cooperativity and homotropic effect but also demonstrates the behavior is changed with H_2O_2 concentrations so that the two sequential homotropic behaviors lead to increased activity. **d** Hill plot in the presence of coamoxiclav. The same effect can be observed here moreover the slope of second line in the presence of coamoxiclav, is more than of ampicillin. The obtained values of $CL_{\max}$, $S_{\max}$ and Hill coefficients have been summarized in Table 1

parameter to describe the salient feature of the sub-system that would be used for the predictive purposes [14].

Hill plot (Fig. 2c) not only confirms the positive allosteric effect (the slope of the lines are more than 1, $m > 1$) but also shows that there are two sequential positive allosteric effects ($m_1 = 1.8$, $m_2 = 3.7$) with increasing of H_2O_2 concentration (Table 1). In Fig. 2d it has been demonstrated that calculated initial velocities (V-cal) has

obtained from Hill equation (see “Materials and Methods” section) is in a good coincidence with experimental initial velocity (V_{exp}).

As it is obvious in Fig. 3; both ampicillin and coamoxiclav are promoter of Hp–Hb peroxidase activity (heterotropic effect or heteroactivation), but they have not changed the sigmoidal shape of the saturation curve (homotropic effect or autoactivation). It seems that both drugs induce a new conformation to the complex which makes it more active. In another word, auto-activation and hetero-activation are occurred simultaneously.

Analysis of the Hp–Hb complex saturation curves (Fig. 3) has shown that Hp(2-2)–Hb complex maintains homotropic behavior of its peroxidase activity in presence of mentioned antibiotics. Moreover, antibiotics have not changed the first Hill coefficient, while the second Hill coefficient has decreased in the presence of ampicillin and increased in the presence of coamoxiclav (Fig. 4c, d respectively). These results have been summarized in Table 1. Comparison of amounts of CL_{max} shows that respective drugs increase CL_{max} of complex to remove H_2O_2 (Fig. 4b; Table 1). This effect is more reasonable in the presence of ampicillin rather than coamoxiclav (about 29% for ampicillin and 7% for coamoxiclav). Although, S_{max} has been shifted to relatively higher concentrations of H_2O_2 in the presence of coamoxiclav, but ampicillin has not any considerable effect on S_{max} (Fig. 4b; Table 1). S_{50} has not been changed in the presence of drugs (Table 1).

In conclusion, ampicillin and coamoxiclav are able to increase antioxidant property of Hp(2-2)–Hb complex via the heterotropic effect on the complex peroxidase activity. It is reasonable to note that these two drugs are usually used under pathologic condition caused by infectious diseases. Also it is resulted that haptoglobin is an acute phase protein [15, 16]. Furthermore, in vitro studies show that two antibiotics (especially ampicillin) may help Hp–Hb complex to remove hydrogen peroxide from serum. We used tetraguaiacol as a second substrate of the enzyme instead of vitamin C (ascorbic acid) which is a non-enzymatic antioxidant. Our study suggests that in individuals with Hp2-2 which consume above mentioned antibiotics due to the infectious fever, the antioxidant activity of Hp–Hb complex increases when they use more vitamin C. Despite of our previous study which was carried out at the physiological temperature, the current study has been performed at the pathological temperature [23]. This study would be beneficial to improve the efficiency of Hp–Hb complex to remove hydrogen peroxide from serum under oxidative stress condition in presence of two mentioned antibiotics especially ampicillin. To complete our conclusion, we are trying to expose our in vivo studies in the early future.

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References

1. Delanghe JR, Langlois MR. Haptoglobin polymorphism and body iron stores. *Clin Chem Lab Med*. 2002;40:212–6.
2. Sadrzadeh SM, Graf E, Panter SS, Hallaway PE, Eaton JW. Hemoglobin a biologic fenton reagent. *J Biol Chem*. 1984;259:14354–6.
3. Vickers AE, Sinclair JR, Fisher RL, Morris SR, Way W. Blood cell oxidative stress precedes hemolysis in whole blood-liver slice co-cultures of rat, dog, and human tissues. *Toxicol Appl Pharmacol*. 2010;244:354–65.
4. Cooper CE, Silaghi-Dumitrescu R, Rukengwa M, Alayash AI, Buehler PW. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of hemoglobin-based oxygen carriers. *Biochim Biophys Acta*. 2008;1784:1415–20.
5. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem*. 1996;42:1589–600.
6. Van Vlierberghe H, Langlois M, Delanghe J. Haptoglobin polymorphisms and iron homeostasis in health and in disease. *Clin Chim Acta*. 2004;345:35–42.
7. Alfson A, Waks M. The hemoglobin binding and activation by haptoglobins: spectrophotometric and potentiometric measurements. *Biochem Biophys Res Commun*. 1966;23:62–7.
8. Adams EC, Weiss MR. Calorimetric studies of the haemoglobin–haptoglobin reaction. *Biochem J*. 1969;115:441–7.
9. Chiancone E, Alfson A, Ioppolo C, Vecchini P, Agrò AF, Wyman J, Antonini E. Studies on the reaction of haptoglobin with haemoglobin and haemoglobin chains. I. Stoichiometry and affinity. *J Mol Biol*. 1968;34:347–56.
10. Laurell CB. Purification and properties of different haptoglobins. *Clin Chim Acta*. 1959;4:79–81.
11. Nagel RL, Gibson QH. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J Biol Chem*. 1971;246:69–73.
12. McCormick DJ, Atassi MZ. Hemoglobin binding with haptoglobin: delineation of the haptoglobin binding site on the α -chain of human hemoglobin. *J Protein Chem*. 1990;9:735–42.
13. Theilgaard-Mönch K, Jacobsen LC, Nielsen MJ, Rasmussen T, Udby L, Gharib M, Arkwright PD, Gombart AF, Calafat J, Moestrup SK, Porse BT, Borregaard N. Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules, and released by neutrophils in response to activation. *Blood*. 2006;108:353–361.
14. Connell GE, Smithies O. Human haptoglobins: estimation and purification. *Biochem J*. 1959;72:115–21.
15. Melamed-Frank M, Lache O, Enav BI, Szafrank T, Levy NS, Ricklis RM, Levy AP. Structure-function analysis of the antioxidant properties of haptoglobin. *Blood*. 2001;98:3693–8.
16. Pavlíček Z, Jaenicke R. On the mechanism of hemoglobin–haptoglobin complex formation. *Eur J Biochem*. 1971;18:305–12.
17. Houston JB, Kenworthy KE. In vitro-in vivo scaling of cyp kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metab Dispos*. 2000;28:246–54.
18. Polonovski M, Jaly MF. Existence dans le plasma sanguin d'une substance activant l'action peroxydasique de l'hémoglobine. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales*. 1938;129:457–60.
19. Polonovski M & Jaly MF. Preparation of a new fraction of the plasma proteins, haptoglobin. *Comptes Rendus de l'Academie des Sciences* 1940;211:517–9.

20. Balestrieri M, Cigliano L, De Simone ML, Dale B, Abrescia P. Haptoglobin inhibits lecithin-cholesterol acyltransferase in human ovarian follicular fluid. *Mol Reprod Dev.* 2001;59: 186–91.
21. Cigliano L, Spagnuolo MS, Dale B, Balestrieri M, Abrescia P. Estradiol esterification in the human preovulatory follicle. *Steroids.* 2001;66:889–96.
22. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. *Nature.* 2001;409:198–201.
23. Tayari M, Moosavi-Nejad SZ, Shabani A, Rezaei-Tavirani M, Dehghan Shasaltaneh M. Heterotropic effect of β -lactam antibiotics on antioxidant property of haptoglobin(2-2)-hemoglobin complex. *Iran J Pharm Res.* 2011;10:313–20.