# CHAPERONE-LIKE ACTIVITY OF HUMAN HAPTOGLOBIN: SIMILARITY WITH α-CRYSTALLIN

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Human haptoglobin (Hp) has been shown to have chaperone-like activity in preventing thermally induced aggregation of catalase and γ-crystallin. No differences in the chaperone-like behaviour of genetic types Hp 1-1 and a mixture of types Hp 2-1 and Hp 2-2 (*i.e.* Hp II) were found. Haptoglobin not only suppresses heat-induced aggregation of proteins but also prevents γ-crystallin from aggregation by oxidative stress. In addition, haptoglobin also provides protection against glycation-induced inactivation of catalase by glyceraldehyde. Chaperone-like activity of haptoglobin decreases in the course of its glycation. Refolding studies have shown that Hp exhibits its chaperone-like activity predominantly on the unfolding and not on the refolding pathway. Although Hp and α-crystallin have no sequence similarities, it seems that their chaperone-like activities are of the same type.

**Key words:** Proteins; Glycoproteins; Human haptoglobin; Chaperones; α-Crystallin; Glycation; Catalases.

α-Crystallin is the protein of the vertebrate ocular lens. Believed to be strictly a lens-specific protein until recently; α-crystallin has now been found in many non-lenticular tissues[1]. Another important recent development is the finding that α-crystallin shares extensive structural and functional similarities with small heat-shock proteins (sHsps) and its biosynthesis is inducible by heat and other stress conditions[2]. A new light on a potential physiological role of sHsps is general and α-crystallin in particular has been shed by a recent discovery that α-crystallin acts *in vitro* as a chaperone by preventing the aggregation of proteins denatured by heat or other insults. The chaperone activity of α-crystallin was first reported by Horowitz[3] and quickly confirmed by others[4]. This observation has been subsequently extended to other sHsps (ref.[5]). It is believed that α-crystallin as molecular chaperone protects other damaged lens proteins from denaturation and aggregation, thereby preventing formation of the light scattering

centres and opacification of the lens in cataract[6]. The chaperone function of α-crystallin and related sHsps is likely to be imported into other tissues.

In common with other chaperones, α-crystallin appears to bind non-native proteins forming a relatively stable complex. The results of Das *et al.*[7] indicated that α-crystallin is significantly different from other chaperones in that it does not recognize in its native conformation the folding intermediates formed during proteins refolding reaction *in vitro* (ref.[8]). The substrate specificity of α-crystallin appears to be limited to specific non-active intermediates that occur on the denaturation pathway only.

More evidence has accumulated for the chaperone-like activity of α-crystallin in recent years. α-Crystallin not only suppresses heat-induced aggregation of lens proteins, but also that of non-lenticular proteins such as alcohol dehydrogenase, citrate synthase[5] and carbonic anhydrase[9]. α-Crystallin also prevents γ-crystallin from aggregation by UV radiation and oxidative stress[4]. α-Crystallin provides protection against sugar-induced inactivation of glucose-6-phosphate dehydrogenase[10]. In addition, α-crystallin was shown to protect catalase against steroid-induced inactivation[11] and 6-phosphogluconate dehydrogenase against inhibition by carbamylation[12]. Our understanding of molecular basis of chaperone activity is still far from complete.

The fact that observable raised haptoglobin (Hp) levels are found in all inflammatory diseases, in most of the patients with cancer and in association with stress[13] brought us to the idea, that acute phase protein – haptoglobin – might also exhibit chaperone properties. Haptoglobin is a blood protein from the group of $\alpha_2$-glycoproteins which shows an outstanding ability to form very rigid complexes with hemoglobin[14,15] (Hb). The physiological role of blood glycoproteins including blood haptoglobin is intensively studies. In the case of haptoglobin the formation of its complex with Hb prevents hemoglobin from being eliminated *via* the kidney. Thus the kidneys are protected and iron for the living body is saved[16]. It was suggested that haptoglobin might also act as antioxidant *in vivo*, protecting against heme-stimulated oxygen radical damage[17].

The present paper, dealing with human haptoglobin in more detail, reports the chaperone-like properties of Hp and compares these properties with the behaviour of stress protein α-crystallin.

## EXPERIMENTAL

*Chemicals*. All the reagents and chemicals in these experiments were of analytical grade. Bovine catalase and D,L-glyceraldehyde (GCA) were purchased from Sigma Chemicals Co. Urea, sodium hydrogenphosphate and sodium dihydrogenphosphate were products of Lachema Brno, hydrogen peroxide from Chemapol, Prague.

*Protein isolation*. γ-Crystallin was prepared according to the method of Björk[18] from the cow lenses in the Nuffield Laboratory of Ophthalmology at Oxford University. For the isolation of bovine lens proteins the supernatant was loaded onto a Sephadex G-50 gel filtration column.

Haptoglobin type II (Hp II) was isolated from the Cohn's fraction IV of human blood serum (Immuna, Šarišské Michaľany, Slovak Republic) by precipitation with ammonium sulfate and chromatography on DEAE cellulose at pH 5.0, using a method devised in this laboratory[14].

Haptoglobin 1-1 (Hp 1-1) was isolated from human serum containing only the genetic type 1-1 in the Laboratory for Toxicology and Judical Chemistry, Faculty of Medicine, Charles University, Prague[14].

*Chaperone activity assay*. Thermally induced aggregation of proteins was measured on a Cecil 8020 spectrophotometer. The apparatus was connected with a CE 245 heater to regulate the corresponding temperature of quartz cells with an accuracy of ±0.3 °C. The mixture of proteins and haptoglobin was heated at corresponding temperature and the apparent optical density, OD, due to light scattering at 360 nm was followed as a function of time.

*Gel filtration*. A protein mixture of γ-crystallin and Hp II was incubated in 100 mM phosphate buffer (pH 7.4) for 60 min at 60 or 70 °C. The incubated samples were filtered with Millipore 0.45 μm membrane filters and the soluble proteins were analyzed by HPLC. HPLC Thermo Separation Production System was used in conjunction with a Watrex 250 × 8 mm G60 C65 exclusion column. All analyses were performed at ambient temperature. The mobile phase was 100 mM phosphate buffer, pH 7.4. An isocratic flow rate of 1 ml/min was used. Protein elution was followed by absorbance of the eluate at 214 nm.

*Unfolding and rapid refolding of γ-crystallin*. A sample of γ-crystallin was dissolved in 20 mM phosphate buffer (pH 7.4) with 8 M urea and equilibrated for 5 h at room temperature. Rapid refolding was achieved by diluting 50 μl of this sample with 950 μl of 20 mM phosphate buffer (pH 7.4) free of denaturant. The refolding buffer was prepared with 100 mM NaCl. The optical density of the solution was measured at 360 nm.

*Glycation of catalase*. An enzyme solution with 0.15 mg/ml of catalase and 1 mM GCA in a 0.05 M phosphate buffer (pH 7.4) was incubated for 6 h at 37 °C. At the times of 0, 2, 4 and 6 h, a sample of the incubated solution was taken and after dilution, the enzyme activity of catalase was measured according to the standard procedure given by Sigma[19].

*Glycation of haptoglobin*. A reaction mixture, consisting of 2 mg/ml Hp II, phosphate buffer (pH 7.4) and $1.25 \cdot 10^{-4}$ M GCA, was incubated at 37 °C. At incubation times 0, 4 and 24 h, 0.1 ml of this solution was added to 0.9 ml of phosphate buffer and 1 ml of catalase solution (0.3 mg/ml). The time dependence of $OD_{360}$ of this solution at 50 °C was measured. The corresponding time dependence of catalase in phosphate buffer without GCA was measured as reference.

*Oxidation of γ-crystallin*. An amount of 0.3 mg/ml of γ-crystallin was incubated for 4 h at 37 °C in a reaction mixture, consisting of 0.06 M phosphate buffer pH 7.0, $2.5 \cdot 10^{-4}$ M sodium ascorbate, $5 \cdot 10^{-5}$ M $FeCl_3$, $1.5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M $H_2O_2$. Hydroxyl radicals and

probably superoxide anion radicals ($O_2^{-\bullet}$) were available as oxidants[4]. The optical density at 360 nm was measured every 30 min starting at the zero time of oxidation.

## RESULTS AND DISCUSSION

### *Chaperone-Like Activity*

The chaperone-like activity of human haptoglobin Hp II was assessed by determining its ability to prevent the heat-induced aggregation of catalase at 50 °C and that of γ-crystallin at 55 °C.

The effect of Hp II on thermal aggregation of catalase was clearly demonstrated (Fig. 1). Heated samples of catalase containing haptoglobin exhibited marked decrease in optical density in comparison with catalase alone. Maximum protection was shown to occur at the molar ratio catalase/Hp 1 : 4. Further addition of Hp did not change the achieved maximum protection any more.

γ-Crystallin was chosen for thermal aggregation as an example for a lens protein which is in the organism protected by α-crystallin acting as a molecular chaperone. The protecting role of Hp II on thermal aggregation of

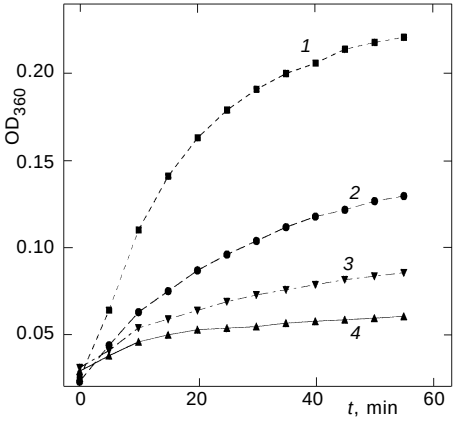


FIG. 1
The effect of haptoglobin on thermal aggregation of catalase upon heating at 50 °C. Catalase (0.15 mg/ml) was incubated alone (*1*), at a catalase/Hp II molar ratio of 1 : 1 (*2*), 1 : 2 (*3*) and 1 : 4 (*4*). Aggregation was monitored by measuring of optical density at 360 nm

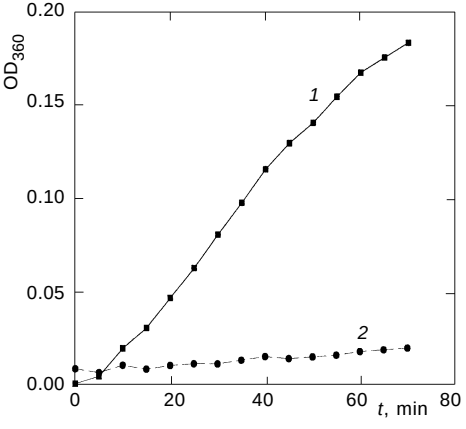


FIG. 2
The effect of haptoglobin on thermal aggregation of γ-crystallin upon heating at 55 °C. γ-Crystallin (1.0 mg/ml) was incubated alone (*1*) and at a γ-crystallin/Hp II molar ratio of 10 : 1 (*2*). Aggregation was monitored by measuring of optical density at 360 nm

γ-crystallin is demonstrated in Fig. 2. The observed decreases in $OD_{360}$ for γ-crystallin in the presence of Hp II were much stronger when compared to the experiments with catalase. Full protection was alrready reached at a molar ratio γ-crystallin/Hp II of 10 : 1 compared with 1 : 4 in the case of catalase.

All these experiments were repeated with Hp 1-1. The results showed no difference in the ability to prevent heat-induced aggregation of the used proteins.

*Rapid Refolding of γ-Crystallin*

In order to get a better insight into the chaperone-like activity of Hp II, the refolding properties of γ-crystallin were investigated. The refolding method is widely used to study refolding properties of proteins, where proteins in unfolded state are rapidly transferred to the conditions that favour their folding.

Rapid refolding experiments show, that the presence of Hp II in the refolding buffer marginally increases the recovery of γ-crystallin in the soluble form (see Fig. 3). The turbidity of γ-crystallin after rapid refolding into the buffer with Hp II is only reduced by approximately 30%. Haptoglobin itself remains clear upon rapid refolding in the concentration range studied. The still very high turbidity shows that in the case of rapid refolding the protection ability of haptoglobin is substantially reduced. On the basis of this result, it was concluded that native Hp has substrate specificity different from other chaperones and recognize specific non-native intermediates formed on the denaturation pathway, predominantly with low affinity

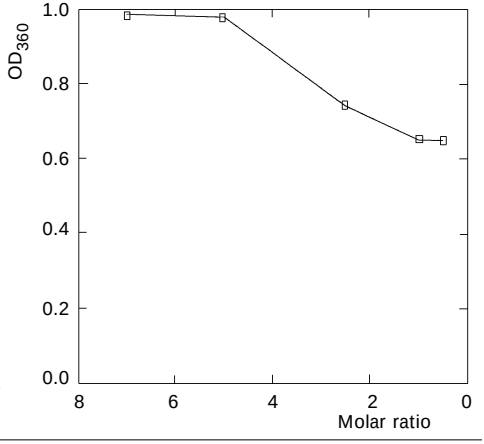


FIG. 3
Refolding of γ-crystallin in the presence of native haptoglobin. Turbidity was plotted as a function of the molar ratio γ-crystallin/haptoglobin. The optical density was measured at 360 nm

for intermediates formed on the refolding pathway. A similar behaviour was observed by Raman *et al.*[20] with α-crystallin as a chaperone-like protein.

*Inhibition in the Process of Enzyme Glycation*

Glycation-induced modification of structural proteins has been widely studied and is thought to play a major role in ageing, diabetes and cataract[20]. The aldehyde groups of sugars react with the amino groups of proteins forming adducts of the Schiff base type and Amadori products.

The sugar does not cause instant inhibition, but inactivates in a slow, time-dependent fashion, consistent with inhibition caused by chemical modification of the enzyme by glycation. Figure 4 shows the time dependence of catalase activity in the course of glycation by GCA. The presence of Hp in the incubated solution decreases the rate of activity inhibition. The same observation was obtained by Ganea and Harding[10] with α-crystallin and glucose-6-phosphate dehydrogenase, a system important in the development of diabetic complications.

Glycation of Hp II itself reduces the ability of Hp to protect catalase against heat-induced aggregation. After haptoglobin was glycated for 24 h, we measured a loss of protection ability (about 30% compared with haptoglobin unglycated) in the course of heat-induced aggregation of catalase. However, the protecting function of Hp is not completely lost in the glycation process (Fig. 5).

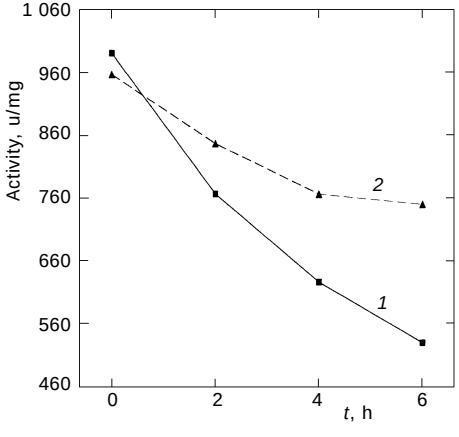


FIG. 4
Time-dependance of the enzyme activity in the course of catalase glycation by GCA in the presence of haptoglobin. Enzyme activity of catalase was measured for catalase alone (*2*) and for a catalase/Hp II molar ratio of 1 : 4 (*1*)

*Inhibition in the Process of Crystallin Oxidation*

There is substantial evidence that oxidative stress is an initiating or early event in the development of various diseases, *e.g.* cataract[21]. It has been found that patients with cataract have elevated levels of $H_2O_2$. The observed concentrations of $H_2O_2$ are capable of causing cataract in organ culture and there is extensive oxidative damage to lens proteins in patients with cataract. The investigation of Wang and Spector[4] indicated that α-crystallin can act as a chaperone under conditions of oxidative stress, decreasing the light scattering of other crystallins. Because oxidative stress is thought to be present under normal physiological conditions, it is probable that α-crystallin contributes to the mechanism that maintains the lens in a transparent state. In our study, comparing the chaperone-like properties of Hp with α-crystallin, it is of interest to determine whether the haptoglobin chaperone-like activity is effective under conditions of oxidation. Figure 6 clearly shows that in the presence of marked oxidative stress, Hp can mini-

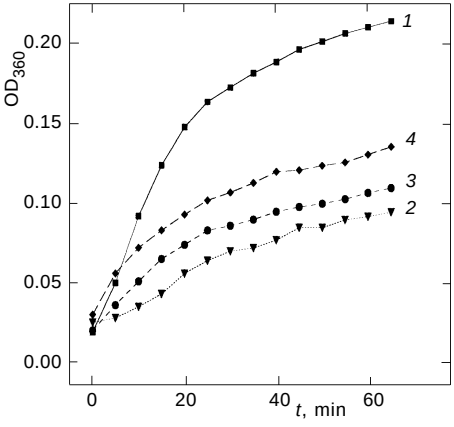


FIG. 5

The effect of glycation of haptoglobin on its ability to prevent catalase thermal aggregation. Hp II (2 mg/ml) was glycated with $1.25 \cdot 10^{-4}$ M glyceraldehyde at 37 °C. Thermal aggregation of catalase was measured over a period of 60 min at 50 °C for 0.15 mg/ml catalase alone (*1*) and for a catalase/Hp II molar ratio of 1 : 2 after a glycation period for haptoglobin of 0 h (*2*), 4 h (*3*) and 24 h (*4*). Aggregation was monitored by measuring of optical density at 360 nm

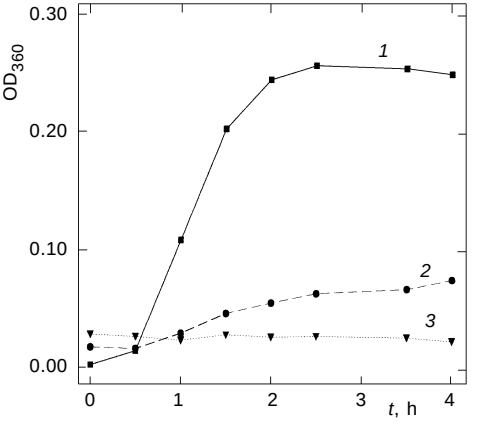


FIG. 6

The effect of haptoglobin on oxidation-induced aggregation of γ-crystallin at 37 °C. The oxidation of γ-crystallin (0.3 mg/ml) with $2.5 \cdot 10^{-4}$ M sodium ascorbate, $5 \cdot 10^{-5}$ M $FeCl_3$, $1.5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M $H_2O_2$ was measured for γ-crystallin alone (*1*) and for a γ-crystallin/Hp II molar ratios of 10 : 1 (*2*) and 5 : 1 (*3*). Aggregation was monitored by measuring of optical density at 360 nm

mize protein aggregation and high scattering within the measured time. The protection capacity of Hp is nearly the same as in the case of heat-induced denaturation.

*Complex Formation and HPLC Analyses*

After heating, mixtures of γ-crystallin and Hp were initially chilled and then analyzed on a gel filtration column. Figure 7 shows an overlay of the elution patterns for an unheated control (curve *1*) and for samples heated for 60 min at 60 °C (curve *2*) and 70 °C (curve *3*), respectively. The non-denaturated control sample (curve *1*) showed peaks for Hp followed by γ-crystallin.

The elution patterns for the heat denaturated samples at 60 °C showed only the reduction of the Hp peak. Most striking was the change of the γ-crystallin and Hp peak, after heating at 70 °C (curve *3*). Concomitant with the decrease in the Hp and γ-crystallin peaks, we observed the generation of new chromatographic components, eluting before the Hp position. These components correspond to the soluble high-molecular-weight complex ($HMW_C$) formation after chaperone interaction, *i.e.*, interaction between Hp and γ-crystallin. The samples of Hp and γ-crystallin, respectively, heated separately at 70 °C, exhibited neither aggregation nor precipitation in the case of Hp and remarkable precipitation for γ-crystallin. Similar observations were obtained by Wang *et al.*[4] and Rao *et al.*[22] in the study of the α-crystallin–γ-crystallin system.

α-Crystallin and Hp as chaperone-like proteins seem to have different substrate specifity as compared with other chaperones. Thus, proteins such

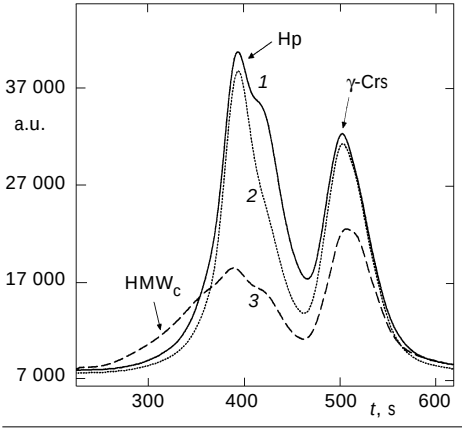


FIG. 7
The effect of thermal denaturation on the elution profile of the haptoglobin (Hp) and γ-crystallin (γ-Crs) from HPLC gel filtration column and formation of high-molecular-weight components ($HMW_C$). Profile *1* was for a non-denaturated control sample and profiles *2* and *3* correspond to samples heated at 60 and 70 °C, respectively

as GroEL, HSP70, HSP90 or the SecB/SecA system are believed to constitute an integral part of protein folding and/or protein translocation machinery *in vivo* (ref.[23]). Their mechanism of action involves trapping of early folding intermediates and subsequent release of the substrate in an ATP-dependent fashion.

This is in contrast with α-crystallin and Hp, which appear to have no ATP-ase activity and are unlikely to be part of a general folding machinery. Recently, however, the enhancement of αB-crystallin chaperone function by ATP was described[24]. The detailed research of both α-crystallin and Hp in this field is necessary in the next future. The chaperone-like function of Hp, α-crystallin (and presumably of other heat shock proteins) is likely to be of considerable physiological significance. With its high capacity to prevent aggregation of states and acute phase responses.

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