

Alpha-crystallin acting as a molecular chaperone protects catalase against steroid-induced inactivation

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Abstract A link between corticosteroid therapy and the development of cataract has been known for many years. However, the precise underlying molecular mechanism of pathology has not been characterised, although a role for direct deleterious interactions between corticosteroids and lenticular proteins has been investigated. α -Crystallin is a major lens protein that has exhibited chaperone properties *in vitro*. Catalase is a ubiquitous enzyme that is an important scavenger of hydrogen peroxide *in vivo*. The corticosteroid prednisolone-21-hemisuccinate was found to inactivate bovine liver catalase, *in vitro* in a progressive manner. Coincubation of α -crystallin with catalase in a 1:2 molar ratio (one α -crystallin to two catalase molecules) fully protected against this inactivation. The protection was specific. Aspirin-like analgesics, putative anti-cataract drugs offered no such protection.

Key words: Catalase; Prednisolone-21-hemisuccinate; α -Crystallin; Steroid-induced cataract; Chaperone

1. Introduction

Catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9) break down the potentially harmful hydrogen peroxide in cells [1]. Loss of activity of one of these enzymes could leave tissues vulnerable to peroxide induced damage. In human cataract and in experimentally induced cataracts the activities of some important enzymes, including catalase fall [2,3]. Evidence that steroids could induce cataract has accumulated since Black et al. observed that rheumatoid arthritis patients administered oral corticosteroids had a significant association with the development of posterior subcapsular cataract [4]. Such conclusions have been confirmed in various epidemiological studies [5–7]. Steroid-induced cataracts have been observed in rabbit [8] and chick embryo [9] models after injection with corticosteroids. Glucocorticoid protein adducts have been observed in human lenses of cataract patients who had previously undergone glucocorticoid therapy and in rat lenses incubated with prednisolone [10].

A mechanism by which a corticosteroid could inactivate an enzyme would be via an attack by a reactive carbonyl group at C-20 on a free α -amino group or the ϵ -amino groups of lysine residues. Prednisolone-21-hemisuccinate contains such a reactive carbonyl group. After formation of a Schiff base, a rearrangement to a more stable Heyns product could occur which would contain another reactive carbonyl group [8]. Catalase, although its N-terminus is blocked, contains 92 lysine residues per tetramer. Seventy two of these groups are exposed on the surface of the molecule [11], some of these may be susceptible to post translational modification. The

level of glucose in the lens increases in steroid induced cataract [9]. Thus problems involved with high steroid concentrations *in vivo* could be augmented by sugar-induced attack of lens proteins.

α -Crystallin, a major lens protein, has sequence homology to the small heat shock proteins [12] and exhibits chaperone activity *in vitro* [13]. The first evidence of a chaperone role for α -crystallin came from the observation by Horwitz that α -crystallin could suppress the thermally-induced aggregation of β_L and γ crystallin and that of a variety of enzymes [14]. Evidence for additional physiological roles for α_A and α_B , the subunits for α -crystallin, besides that of maintaining the structural integrity of the lens has come from the finding that their expression is not confined to the lens [15–17]. Elevated levels of α_B crystallin mRNA or protein are associated with a variety of pathological conditions [16,18]. More recently, this protein has been shown to fully protect against glycation-induced inactivation of glucose-6-phosphate dehydrogenase [19]. This is consistent with a chaperone function. α -Crystallin has not been crystallised, so its structure/function relationship remains to be elucidated. Several models for the quaternary structure have been suggested [20–23].

Aspirin-like analgesics, including aspirin, delayed the onset of diabetic cataract in rats [24,25]. Epidemiological studies have shown a reduced risk of cataract associated with regular intake of non steroidal anti-inflammatory drugs [5,26,27]. Aspirin and ibuprofen have also been shown to significantly reduce potentially deleterious modifications of lens proteins *in vitro*, such as glycosylation [28].

In the results presented we show that prednisolone-21-hemisuccinate inactivates catalase *in vitro*. α -Crystallin fully and specifically protected against this inactivation over several days in a 1:2 molar ratio to that of catalase. The aspirin-like analgesics did not offer any significant protection against steroid-induced inactivation of catalase.

2. Materials and methods

2.1. Materials

α -Crystallin was isolated from bovine lenses by gel chromatography on Sephacryl S300 HG using the method of Slingsby and Bateman [29]. Bovine liver catalase, egg lysozyme, bovine serum albumin, prednisolone-21-hemisuccinate and all other proteins and chemicals were obtained from Sigma, Poole, Dorset, UK.

2.2. Incubation

Catalase (523 units, 24.9 μ g) was incubated in a shaking water bath in 50 mM sodium phosphate buffer, pH 7 with and without 1 mM and 2 mM prednisolone-21-hemisuccinate for between 1 and 6 days in a final volume of 1 ml.

In some experiments, α -crystallin, bovine serum albumin, lysozyme, human albumin, egg albumin, α -chymotrypsinogen, ribonuclease and glucose oxidase (each at 20 μ g/ml) were added to different vials during the incubation. Alternatively aspirin, paracetamol and ibuprofen

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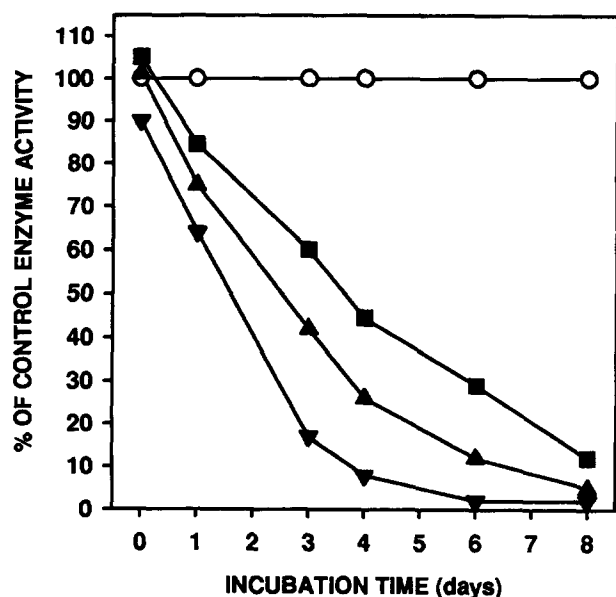


Fig. 1. Inactivation of catalase in the absence (○) and presence of 1 mM (■), 2 mM (▲) and 5 mM (▼) prednisolone-21-hemisuccinate.

(each at 10 mM) were added to different vials. Each solution was then inoculated through sterile 0.2 μ m Nalgene syringe filters into sealed, autoclaved sterile vials.

2.3. Assays

Enzyme activity was assayed by monitoring the decreased absorbance at 240 nm resulting from the decomposition of hydrogen peroxide for 1 min at 37°C. The assay mixture consisted of 2.5 ml of H_2O_2 solution from a stock solution of 0.1 ml of 30% H_2O_2 diluted in 50 ml of sodium phosphate buffer, pH 7, and 20 μ l of the incubation solution, made up to a final volume of 3 ml with the phosphate buffer. All assays (3.5 units, 0.17 μ g) were carried out in triplicate. Activity is expressed relative to the control activity at each respective time interval, set at 100%, unless otherwise stated.

2.4. Fluorescence

Incubated samples were dialysed extensively against 50 mM sodium phosphate buffer, pH 7 and 800 μ l of the sample was excited at 295 nm and measured for tryptophan fluorescence, slit width 10 nm, emission spectra of 300 to 500 nm, on a Perkin Elmer LS 50 B luminescence spectrometer.

2.5. High-pressure liquid chromatography

Incubation solutions (2 ml) were dialysed against volumes of 100 ml 50 mM phosphate buffer and 1 vol. 100 ml distilled water at 1 h intervals over 4 h. 1.5 ml of each solution was then freeze dried, samples were reconstituted in 50 μ l of distilled water and 30 μ l was then loaded onto a Biosep S4000 size-exclusion column (Phenomenex, Macclesfield, UK) and eluted at 0.5 ml/h. Absorption at 280 nm was followed and 1 min fractions were collected, 400 μ l of which were assayed for catalase activity as described above.

2.6. Statistical analysis

All results were subjected to a Student's *t*-test where relevant.

3. Results

The effect of the glucocorticoid prednisolone-21-hemisuccinate on catalase activity was followed. Prednisolone-21-hemisuccinate inactivated catalase in a slow, steady, progressive manner (Fig. 1). There was no immediate inhibition at time zero which clearly indicates that the inactivation was due to slow post-translational modification of catalase by the steroid.

Prednisolone-21-hemisuccinate at concentrations of 1, 2 and 5 mM seemed to inactivate in a dose-dependent manner, but the different rates of inactivation were not found to be significantly different according to a Student's *t*-test. Extensive dialysis of incubation solutions had no effect on the degree of inactivation, suggesting a strong interaction between prednisolone-21-hemisuccinate and catalase. The emission maxima of the catalase solution containing 1 mM prednisolone-21-hemisuccinate did not differ significantly from that of the control solution containing just catalase after up to 8 days of incubation suggesting that no steroid-induced gross conformational change such as dissociation or aggregation had occurred. Rather we would suggest that the site of initial reaction between a prednisolone-21-hemisuccinate and a catalase molecule is likely to be one at or near the active site that would have the result of inactivation with only a limited conformational change being induced. A good candidate for such a modification would be Lys²³⁶ (bovine liver), the most charged residue on the outside of the catalase molecule [11]. It is an unusual residue in that it is found in a hydrophobic environment in the interior of a β barrel domain of catalase. Such a hydrophobic environment one could postulate would stabilise the reaction of a glucocorticoid with a protein.

α -Crystallin protected against the inactivation of catalase by 1 and 2 mM prednisolone-21-hemisuccinate (Fig. 2). If we assume that under native conditions α -crystallin exists as a complex of 800 kDa [30], then 0.05 μ M (40 μ g/ml) α -crystallin fully protected against the inactivation by 1 and 2 mM prednisolone-21-hemisuccinate in a 1:2 molecular weight ratio to that of catalase [1]; i.e. each molecule of α -crystallin protected two molecules of catalase. Only 24% of control activity remained after 4 days of incubation with 1 mM prednisolone-21-hemisuccinate alone, but with 1 mM prednisolone-21-hemisuccinate and α -crystallin, catalase remained fully active at 4 days ($P = 0.001$). After 6 days only 9% of control activity

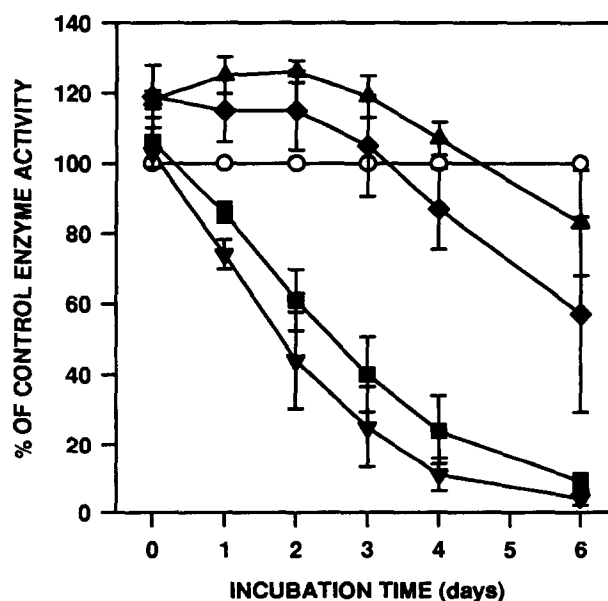


Fig. 2. The % of control enzyme activity of catalase in the absence (○) and presence of 1 mM (■) or 2 mM (▼) prednisolone-21-hemisuccinate was assayed. α -Crystallin was present at 40 μ g/ml in other solutions containing catalase and 1 mM (▲) and 2 mM (◆) prednisolone-21-hemisuccinate that were assayed at the same time intervals over 6 days.

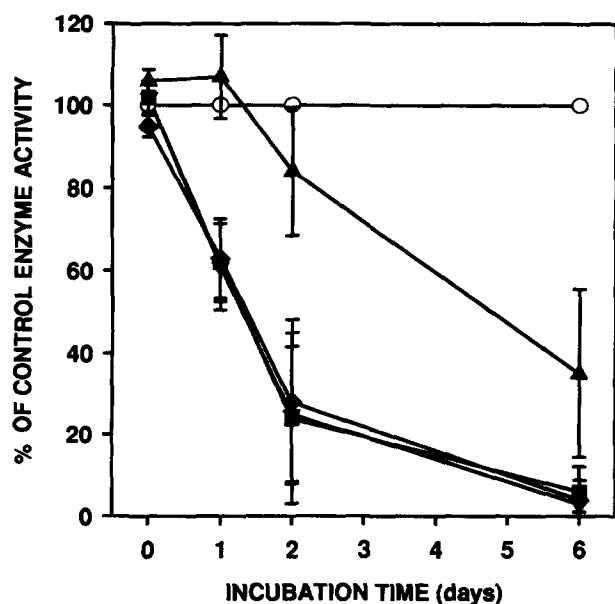


Fig. 3. The % of control enzyme activity of catalase in the absence (○) and presence of 2 mM prednisolone-21-hemisuccinate (■) was assayed. Other incubations including 20 µg/ml of α-crystallin (▲), bovine serum albumin (▼) or egg lysozyme (◆), in addition to catalase and 2 mM prednisolone-21-hemisuccinate were also assayed over 6 days.

remained upon incubation with 1 mM prednisolone-21-hemisuccinate alone, but at this time the addition of α-crystallin conferred the retention of 83% of control activity ($P = 0.021$). Additionally, just 25% of control activity remained after 3 days of incubation with 2 mM prednisolone-21-hemisuccinate, but with 2 mM prednisolone-21-hemisuccinate and α-crystallin, full protection was again conferred to catalase ($P = 0.004$). However, after 6 days only 4% of control activity remained upon incubation with 2 mM prednisolone-21-hemisuccinate, whereas with 2 mM prednisolone-21-hemisuccinate and α-crystallin 57% of control activity remained. This protective effect was found not to be statistically significant ($P = 0.117$). Clearly, α-crystallin could fully protect against inactivation by prednisolone-21-hemisuccinate for a limited period of time. It is of note that none of the previously postulated anti-cataract drugs, aspirin, paracetamol or ibuprofen at the comparatively high concentration of 10 mM, could confer any lasting protection against this inactivation (results not shown).

The protection conferred by α-crystallin was found to be specific. This is shown by incubations of catalase and 2 mM prednisolone-21-hemisuccinate with concentrations of α-crystallin or other proteins at 20 µg/ml, half the w/v concentration of α-crystallin in the previously described experiments. Fig. 3 shows that neither bovine serum albumin nor lysozyme afforded any protection against the inactivation of catalase by 2 mM prednisolone-21-hemisuccinate. After 2 days only 25% of control activity remained in the incubation with 2 mM prednisolone-21-hemisuccinate alone but with 2 mM prednisolone-21-hemisuccinate and α-crystallin, 84% of control activity remained ($P = 0.024$). After 6 days of incubation, only 6% of control activity was found in the solution of 2 mM prednisolone-21-hemisuccinate, however with 2 mM prednisolone-21-hemisuccinate and α-crystallin only 35% of control activity remained which was found not to be significant ($P = 0.316$). It is of note that 10 µg/ml α-crystallin protected

against the inactivation by 2 mM prednisolone-21-hemisuccinate after 1 day ($P = 0.014$), result not shown. Bovine serum albumin (and human albumin) have twice as many lysines per 100 amino acid residues as α-crystallin and so if α-crystallin were protecting against steroid inactivation by non-specifically reacting with these molecules itself, one would expect bovine serum albumin to protect in a similar fashion. In other experiments five other proteins were incubated with catalase instead of α-crystallin. Again no other proteins were able to protect against the steroid-induced inactivation of catalase (Fig. 4). α-Crystallin at 20 µg/ml was able to afford both significant and specific protection for up to 2 days. In summary, α-crystallin at a concentration equal to 0.05 µM significantly and fully protected against steroid inactivation over a period of 4–6 days when incubated with 2 and 1 mM prednisolone-21-hemisuccinate, respectively. 0.025 µM α-crystallin protected to a lesser extent, but protection was statistically significant for at least 2 days. This protection was specific and lower concentrations of α-crystallin conferred very limited protection.

One interesting observation from Figs. 2–4 is that the activities of solutions containing α-crystallin are higher than that of the control, at early incubation periods. This apparent anomaly could be explained by our findings that activity of the control enzyme itself decreases to around 60% of its initial day 0 activity after a period of 6 days. Catalase incubated with α-crystallin alone lost less activity over the same period of time, activity being around 80% of that of the initial control day 0 activity (results not shown). It also appeared that the presence of α-crystallin increases the activity of catalase almost immediately. Therefore, α-crystallin may be able to protect against catalase heat lability at 37°C.

Size-exclusion chromatography was carried out to investigate complex formation. No separate peak indicating a complex of molecular weight greater than that of α-crystallin was observed even after 6 days of incubation (results not shown),

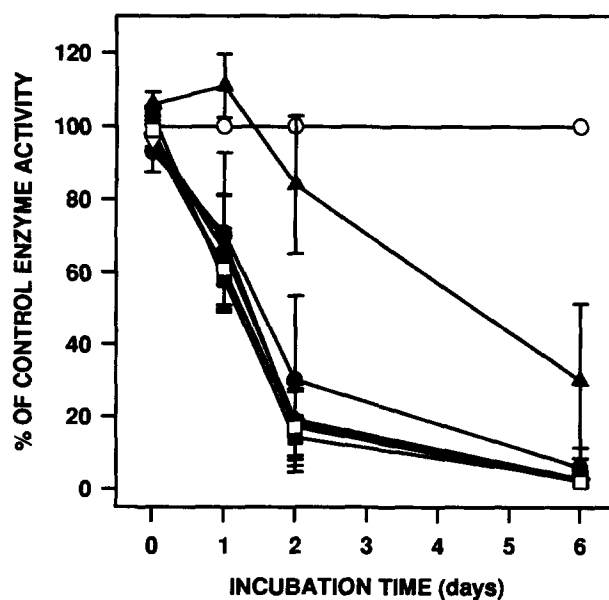


Fig. 4. The % of control enzyme activity of catalase in the absence (○) or presence of 2 mM prednisolone-21-hemisuccinate (■) were assayed. Other incubations including 20 µg/ml of α-crystallin (▲), human albumin (▼), egg albumin (◆), α-chymotrypsinogen (●), ribonuclease (▽) and glucose oxidase (□) with catalase and 2 mM prednisolone-21-hemisuccinate were also assayed over 6 days.

although the possibility of complex dissociation during the isolation process cannot be excluded. To determine whether catalase was protected by forming a stable complex without a significant increase in size of α -crystallin, we measured the catalase activity in the α -crystallin containing fractions. No activity was found in this fraction. Catalase activity was only found in the fractions corresponding to tetrameric catalase. During the 6 days of incubation of prednisolone-21-hemisuccinate and catalase with α -crystallin there was an increase in the size of the α -crystallin peak relative to the catalase peak (results not shown). In incubations carried out without the steroid, this change in relative size was not seen.

4. Discussion

Prednisolone-21-hemisuccinate inactivated catalase. Enzyme levels fall during the onset of steroid-induced cataract [2,3]. All prednisolone-21-hemisuccinate molecules would potentially be reactive and as these molecules are in a large molar excess to catalase molecules in a ratio of at least 10000:1 in our *in vitro* system, any system of protection by a protein would have to be more elaborate than simply that of it reacting with prednisolone-21-hemisuccinate itself. The results presented here offer evidence for the role of α -crystallin as a molecular chaperone *in vitro*. If every lysine residue of α -crystallin reacted with a prednisolone-21-hemisuccinate molecule (at concentrations shown in Figs. 3 and 4) assuming that the lysine residue concentration of α -crystallin was as high as 10 μ M and that all the prednisolone-21-hemisuccinate molecules were in an active state, then there would still be 199 prednisolone-21-hemisuccinate molecules out of every 200 left in solution to react with catalase molecules. Even when prednisolone-21-hemisuccinate was at a concentration of 1 mM, catalase was inactivated rapidly (Figs. 1 and 2). α -Crystallin must therefore protect against steroid induced inactivation of catalase by direct interaction with native or denatured catalase molecules.

The stoichiometry of one molecule of α -crystallin protecting two molecules of catalase against steroid-induced inactivation is consistent with Carver's model of α -crystallin [23]. This model suggests that α -crystallin could have a quaternary structure similar to that of chaperonin 60 (GroEL). The suggested structure consists of two rings of α -crystallin subunits stacked on top of one another with a central cavity. Binding of a catalase molecule could occur within the cavity of each ring with the hydrophobic N-terminal domain of α -crystallin at the centre stabilising such a complex, although steric considerations could argue against an entire catalase molecule fitting in the centre of each ring.

HPLC analysis strongly suggested that α -crystallin did not protect catalase activity by formation of a stable complex with active catalase molecules. No significant formation of a higher molecular weight complex was observed throughout the incubation of catalase, prednisolone-21-hemisuccinate and α -crystallin. The significant increase of the α -crystallin peak relative to that of the catalase peak is of interest. However, the protection could be by a dynamic mechanism.

Whether this protective effect could happen *in vivo* is open to debate, but clearly such an effect would be highly desirable. With *in vitro* non-enzymic glycosylation of lens proteins and in human diabetic cataracts, lens proteins become modified, unfold and aggregate [31,32]. Enzyme activities are reduced [2,3]. This could lead eventually to cataract. The fact that these

changes occur with age and not immediately suggests that a molecule with properties exhibited *in vitro* by α -crystallin must exist. Beswick and Harding [31] have postulated that unfolding of lens proteins by sugars could lead to structures that resemble the molten globule state. Such a scheme could present many substrates for the chaperone properties of α -crystallin.

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