



# Protection of a Restriction Enzyme from Heat Inactivation by $\alpha$ -Crystallin

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**Purpose:** To determine whether the chaperone activity of human  $\alpha$ -crystallin can protect a restriction enzyme from heat inactivation.

**Methods:** The restriction enzyme Nde I was heated in the presence or absence of purified bovine  $\alpha$ -crystallin. Following heat treatment, the enzymatic activity of the heat treated samples was assayed by cleavage of plasmid DNA. The extent of digestion was monitored by agarose gel electrophoresis and visualization of DNA fragments by ethidium bromide staining.

**Results:** Heating of Nde I in the absence of  $\alpha$ -crystallin resulted in inactivation. However, Nde I heated in the presence of  $\alpha$ -crystallin remained active. Furthermore, an increased amount of  $\alpha$ -crystallin provided a longer period of thermal protection.

**Conclusions:** The chaperone activity and thermo-protective effect of  $\alpha$ -crystallin extend to protection of enzymatic activity, not merely the protection from thermally induced aggregation/denaturation. In addition, inclusion of  $\alpha$ -crystallin during some enzymatic reactions may be beneficial.

Predominating the protein profile of the lens are the crystallins, low molecular weight proteins that compose 90% of the mass of the lens [1,2]. Of the three crystallin classes,  $\alpha$ ,  $\beta$ , and  $\gamma$ ,  $\alpha$ -crystallins predominate. Amino acid/DNA sequencing has shown that  $\alpha$ -crystallins are evolutionarily related to small heat shock proteins, a finding that is intellectually satisfying when viewed in relation to long term protein stability in the lens [3-5]. Additionally,  $\alpha$ -crystallins have been demonstrated to have chaperone-like activity in vitro, protecting heterologous proteins from insults [6-14].

The protective effects of  $\alpha$ -crystallin toward heterologous proteins was first demonstrated by the heating of alcohol dehydrogenase (ADH) in the presence and absence of  $\alpha$ -crystallin [6]. At 48 °C, in the absence of  $\alpha$ -crystallin, ADH aggregates and this aggregation can be monitored by light scattering. In the presence of  $\alpha$ -crystallin, heat induced aggregation is eliminated. Numerous other metabolic enzymes were also found to be protected from thermally induced aggregation. The enzymatic activities of the enzymes studied were not reported; inactivation was mentioned although data were not presented.

Subsequent to the initial report by Horwitz, other investigators found that  $\alpha$ -crystallin offered protection from additional insults. Obi and coworkers reported a reduction in UV induced protein aggregation by  $\alpha$ -crystallin [9]. Harding and coworkers reported that  $\alpha$ -crystallin protects catalase from steroid inactivation and 6-phosphogluconate dehydrogenase from carbamylation [7,8,10]. Harding and coworkers also reported protection of enzymatic activity by  $\alpha$ -crystallin, a function not provided by control proteins [7,8,10]. In a separate report that examined aggregation as a measure of protein

denaturation,  $\alpha$ -crystallin was shown to prevent aggregation while enzymatic activity was lost [14]. Thus,  $\alpha$ -crystallin seemed better able to prevent protein aggregation than preserve enzymatic activity. How these two phenomena differ is unknown.

From a protein stability standpoint, the chaperone/thermo-protective activity of  $\alpha$ -crystallin could prove to be useful. In other research areas, in vitro stabilization of proteins includes: stabilization of thermolabile enzymes by trehalose [15] or the heat shock protein dnaK [16], the interaction between sorbitol and lysozyme [17], and the effects of  $\text{Ca}^{2+}$  and sugars on recombinant DNase I [18]. Notable for discussion here is the recognition that some compounds can have appreciable stabilization/survival effects on intact cells and that methods taken to increase intracellular protein stability can have beneficial consequences for higher organisms. For example, recent reports documenting the increased stability of isolated platelets in the presence of antarctic fish antifreeze proteins [19] and the increased survival of heat shock protein expressing cells when ischemically stressed shows how single proteins can perform cellular life saving functions [20,21]. In addition, transgenic plants that produce the protein stabilizing, small molecular weight compound betaine are found to be more resistant to salt and cold temperature stress [22]. Recently,  $\alpha$ B crystallin has also been shown to increase survival of E. coli that are exposed to heat shock [23].

We report a series of experiments designed to test the protective effects of  $\alpha$ -crystallin with respect to enzymatic activity. We have chosen to use readily available, well characterized restriction enzymes as the enzymes to assay for  $\alpha$ -crystallin chaperone activity. Our rationale was that (1) restriction enzymes are completely heterologous to  $\alpha$ -crystallin (2) they are commercially available at low cost (3) their enzymatic activities, including heat inactivation properties are well characterized and (4) they are rapidly and easily assayed for activity. With this system, we have found that the restriction

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enzyme Nde I is protected by  $\alpha$ -crystallin from thermal inactivation. In addition, in the presence of  $\alpha$ -crystallin, at elevated temperatures, Nde I exhibits enhanced enzymatic activity.

## METHODS

**Purification of  $\alpha$ -crystallin:** Total crystallins were extracted from bovine lenses obtained from Alpine Meat (Stockton, CA);  $\alpha$ -crystallin was purified by gel filtration chromatography on a Pharmacia HiLoad 16/60 Superdex 200 column (Pharmacia, Piscataway, NJ). Briefly, decapsulated whole lenses were partially homogenized by stirring in buffer and the resulting cellular suspension disrupted by Dounce homogenization. The solution was centrifuged (15000 rpm, Sorvall SS-34 rotor) and the supernatant retained. The solution was filtered through a 0.2 micron filter and then chromatographed on a SuperDex column connected to a Pharmacia Fast Protein Liquid Chromatography (FPLC) system. High molecular weight  $\alpha$ -crystallin complexes eluting in the void volume were pooled. The majority of  $\alpha$ -crystallin was frozen and stored at  $-20^{\circ}\text{C}$ . Protein concentration was determined using the Pierce BCA kit (Rockford, IL).

Restriction enzymes were purchased from either Life Technologies, Gaithersburg MD or New England Biolabs, Beverly, MA. Enzymes were used with the manufacturers supplied buffer or the buffer indicated in the results. Enzyme activities were used as reported by the manufacturer. Where dilutions were performed, they were performed by dilution of the enzyme into a room temperature solution that was 1x reaction buffer final concentration. Enzymes were not diluted into enzyme storage buffer or any other buffer containing glycerol or other protein stabilizing agents.

Restriction enzymes were assayed by cleavage of plasmid DNA for which the corresponding site was present. Plasmid DNA was prepared by either CsCl ethidium bromide ultracentrifugation or by Qiagen MaxiPrep columns (Qiagen, Santa Clarita, CA). Extent of restriction enzyme cleavage was estimated by ethidium bromide staining of plasmid DNA following electrophoresis on agarose gels.

Incubations of restriction enzyme/ $\alpha$ -crystallin/bovine serum albumin (BSA) mixtures was performed in a Minicycler (MJ Research, Watertown, MA), programmed to the appropriate temperature. Incubations were performed in 500  $\mu\text{l}$  tubes with mineral oil covering the sample during incubation. Usual incubation volumes were 10  $\mu\text{l}$ . Typically, 1  $\mu\text{l}$  of enzyme (20 units of Nde I) was added to a tube containing 1  $\mu\text{l}$  of 10x restriction enzyme buffer, and  $\alpha$ -crystallin (1 or 5  $\mu\text{l}$  of a 10 mg/ml concentrated stock) and water to a final volume of 10  $\mu\text{l}$ . To assay the enzyme activity of the heat treated samples, 1  $\mu\text{l}$  (2 units of Nde I) of the sample was used to digest 200 ng of plasmid DNA in a volume of 10  $\mu\text{l}$  at  $37^{\circ}\text{C}$ .

## RESULTS

**Effect of  $\alpha$ -crystallin on restriction enzyme digestion:** To determine whether the partially purified  $\alpha$ -crystallin had inhibitory effects on restriction digestion of DNA, we assayed several commonly used restriction enzymes for activity in the presence of  $\alpha$ -crystallin. The commonly used restriction en-

zymes, Bam HI, Hind III, Nde I, Pst I and Sst I all remained active in the presence of  $\alpha$ -crystallin. For this experiment, a 5 fold excess of each enzyme (5 units) was incubated for 1 hour with 1  $\mu\text{g}$  of plasmid DNA using manufacturers supplied buffer. Each reaction also contained 1  $\mu\text{l}$  of the purified  $\alpha$ -crystallin (10 mg/ml), final concentration, 1 mg/ml. Each selected enzyme digested the plasmid to completion indicating that  $\alpha$ -crystallin had no major detrimental effects on restriction enzyme digestion (data not shown).

To assay the ability of  $\alpha$ -crystallin to protect restriction enzymes from heat inactivation, we chose the enzyme Nde I. Unlike the other enzymes initially assayed, Nde I can be inactivated by heating. Our initial experiments showed that  $\alpha$ -crystallin at 1 mg/ml protected Nde I from inactivation during a 30 minute,  $50^{\circ}\text{C}$  incubation. Subsequent experiments showed that a higher concentration of  $\alpha$ -crystallin (5 mg/ml) provided protection from inactivation during a 1 hour incubation at  $50^{\circ}\text{C}$  (data not shown).

Hypothesizing that the effect of  $\alpha$ -crystallin could be achieved by any added protein, we performed experiments using  $\alpha$ -crystallin and acetylated BSA as added protective agents. The concentration of BSA in these experiments was recommended by the supplier (100  $\mu\text{g}/\text{ml}$ ).

Lanes 1-3 of Figure 1 show uncut plasmid (control 1), Nde I digested plasmid (control 2) and Xho I digested plasmid (control 3). Lanes 4-6 are restriction fragments produced by different aliquots of Nde I (20 units in a final volume of 10  $\mu\text{l}$ ) subjected to  $50^{\circ}\text{C}$  for 1 hour. Lane 4, shows the results of digestion by Nde I heated with no exogenously added protein; lane 5, Nde I plus added  $\alpha$ -crystallin (5 mg/ml final concentration). The Nde I used in lane 6 was heated in the presence of acetylated BSA (100  $\mu\text{g}/\text{ml}$ ). The pattern of DNA fragments in lane 5 indicates complete digestion. In contrast, lane 6 shows incomplete protection of Nde I from heat inactivation. Lane 6 demonstrates that the recommended concentration of BSA

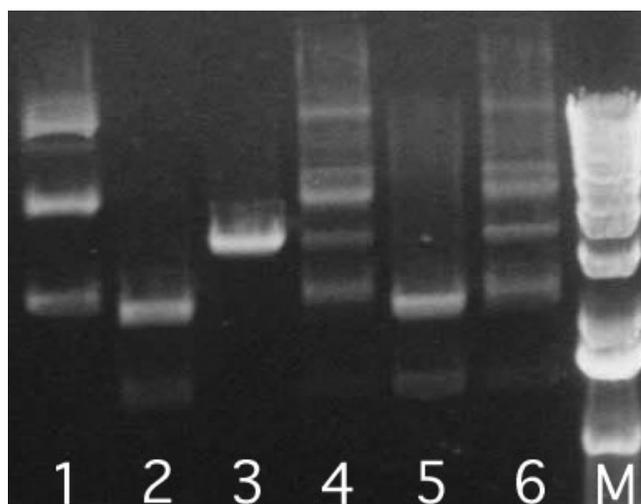


Figure 1. Protection of Nde I from heat inactivation by  $\alpha$ -crystallin and BSA. Lanes 1-3 are controls C1, uncut plasmid; C2, Nde I digested plasmid; C3, Xho I digested plasmid. Lane 4, Nde I incubated without any added protein. Lane 5, Nde I incubated with  $\alpha$ -crystallin. Lane 6, Nde I incubated with BSA. 1 kb ladder DNA standards are in the lane M.

included at the time of heat treatment either provides no protection or protects only a small fraction of Nde I from heat inactivation. Note the small amount of plasmid DNA converted to the linear form (compare to lane 3), and the 1.8 kb fragment indicative of complete digestion (compare to lane 2 or 5). This experiment demonstrates that the thermo-protective effect offered by  $\alpha$ -crystallin is not provided by BSA.

Because the concentration of  $\alpha$ -crystallin in the heat treatment was much higher than the concentration of BSA, we assayed the ability of higher concentrations of BSA to protect Nde I from heat induced inactivation. Contrary to expectations, more BSA in the heat treatment step was not beneficial (data not shown).

With the thermal protective effect of  $\alpha$ -crystallin demonstrated, we examined the effects of  $\alpha$ -crystallin on restriction digestion. In addition to digestion at the normal temperature of 37 °C we performed reactions at the elevated temperature, 50 °C. For these experiments, Nde I was diluted 1:100 into 1x restriction enzyme digestion buffer. The resulting solution, 0.2 units per  $\mu$ l, was used as the restriction enzyme in a series of digestions. Figure 2 shows the results of these digestions.

As in the previous figure, lanes 1-3 show the control reactions C1-3. Lanes 4-9 show the results of digestion of 200 ng of plasmid DNA with 0.2 units of Nde I under different conditions. Lanes 4 and 5 show the restriction enzyme digestion products obtained by digestion with Nde I at 37 °C (lane 4) or 50 °C (lane 5). Lanes 6 and 7 show digestions performed in the presence of 5 mg/ml  $\alpha$ -crystallin; lane 6, 37 °C and lane 7, 50 °C. Lanes 8 and 9 show identical reactions performed in the presence of 1 mg/ml BSA; lane 8, 37 °C and lane 9, 50 °C. When compared to the control reaction C2, only lanes 6 and 7 show a complete digestion pattern. Lane 6 contains a trace of partially digested plasmid DNA while lane 7 shows that the plasmid DNA has been digested to completion. All incubations were for 1 hour, a theoretically appropriate amount of time for 0.2 units of enzyme to digest 200 ng of plasmid DNA. Thus, in the presence of  $\alpha$ -crystallin, Nde I exhibits greater activity at 50 °C than in any other condition assayed.

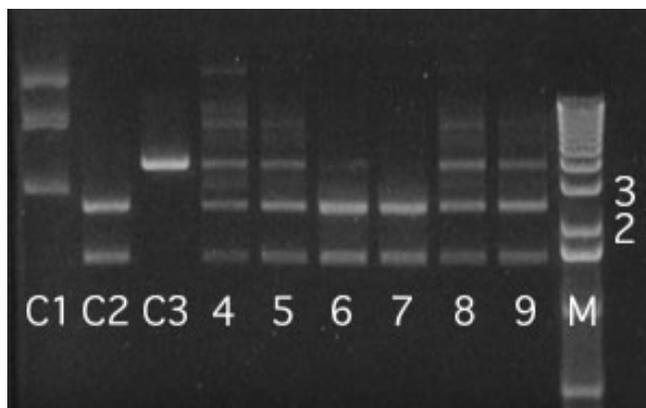


Figure 2. Restriction enzyme digestions performed in the presence of  $\alpha$ -crystallin or BSA. Lanes 1-3, C1-C3. lane 4, Nde I 37 °C; lane 5, Nde I 50 °C, lane 6, Nde I plus  $\alpha$ -crystallin, 37 °C; lane 7, Nde I plus  $\alpha$ -crystallin 50 °C; lane 8, Nde I plus BSA 37 °C; lane 9, Nde I plus BSA, 50 °C. 1 kb ladder DNA standards are in lane M.

## DISCUSSION

We have presented data to show that bovine  $\alpha$ -crystallin acts as a thermal protective agent for the bacterially produced restriction enzyme Nde I. Our data show that  $\alpha$ -crystallin prevents heat induced inactivation of Nde I and, within the experiments described, an increased level of  $\alpha$ -crystallin provides increased protection. An important corollary is that acetylated BSA is unable to provide the same protection, indicating that  $\alpha$ -crystallin is behaving as a chaperone. Our final experiment demonstrates that Nde I is protected from inactivation (and therefore exhibits more activity) in the presence of  $\alpha$ -crystallin at 37 °C and 50 °C. In the context of previous published experiments, these described protective effects add further justification for labelling  $\alpha$ -crystallin a molecular chaperone [6]. Additionally, they suggest a practical use for  $\alpha$ -crystallin.

Following the initial report of  $\alpha$ -crystallin chaperone activity by Horwitz [6], the protective effects of  $\alpha$ -crystallin were investigated using other experimental systems. Harding and coworkers have reported that  $\alpha$ -crystallin protects catalase from steroid induced inactivation [7,8] and 6 phosphogluconate dehydrogenase from carbamylation [10]. In each of these cases, enzymatic activity was monitored and the effect of  $\alpha$ -crystallin was profound. Under conditions which cause 90% inactivation, catalase in the presence of  $\alpha$ -crystallin remains 90% active. In these experiments, the protective effects of  $\alpha$ -crystallin contrasted with the inability of human albumin, egg albumin,  $\alpha$ -chymotrypsinogen, ribonuclease and glucose oxidase to provide any protection. Interestingly, catalase activity in the presence of  $\alpha$ -crystallin is greater than without  $\alpha$ -crystallin, a result similar to one of the observations noted in this report for experiments with Nde I. The activity of 6 phosphogluconate dehydrogenase gave a similar yet not as impressive result. Treatment which inactivated 60% of 6 phosphogluconate dehydrogenase retained 100% of activity in the presence of  $\alpha$ -crystallin. BSA and lysozyme, assayed for their protective effects, initially gave very small increases of enzyme activity but approximately half of the protection provided by  $\alpha$ -crystallin.

Our experiments assess the ability of  $\alpha$ -crystallin to protect a restriction enzyme from thermal inactivation. Initial characterization of purified  $\alpha$ -crystallin revealed two important properties: one, that the presence of  $\alpha$ -crystallin was not inhibitory to restriction enzyme digestion and two, the  $\alpha$ -crystallin fraction we isolated was not highly contaminated with nucleases which would have degraded the plasmid DNA used for activity assays. With a partially purified  $\alpha$ -crystallin fraction exhibiting these characteristics, we could then perform the intended experiments. Although we began our experiments with a number of commonly used restriction enzymes, we concentrated on the less than ideal restriction enzyme, Nde I. This enzyme, as noted in the New England Biolabs catalog, has a half life at 37 °C of 15 minutes. The other enzymes initially assayed displayed heat stability and so were not candidates for thermal protection by  $\alpha$ -crystallin. They have not been assayed for increased cleavage activity at elevated temperatures in the presence of  $\alpha$ -crystallin.

Using purified  $\alpha$ -crystallin, our experiments conclusively show that  $\alpha$ -crystallin can prevent heat induced inactivation of Nde I. As shown in figures 1 and 2, this protection was much greater than acetylated BSA which is often recommended for addition to restriction digestion reactions. While the protein concentrations of  $\alpha$ -crystallin and BSA were not equal, the concentration of BSA used was recommended by the manufacturer or slightly higher. We found that very high concentrations of BSA inhibited Nde I (data not shown). In contrast, at high concentrations, not only does the  $\alpha$ -crystallin not inhibit Nde I, it provides increased thermal protection.

From the onset, we chose to examine preservation of enzymatic activity and not protein aggregation. Monitoring the aggregation of restriction enzymes would have consumed much larger amounts of the enzymes and would have been much more expensive. Studying the enzymatic activity was a more sensitive method to examine the gross effects of heating. While our results do not determine whether or not Nde I aggregated during incubation at 50 °C in the presence of  $\alpha$ -crystallin, we know that if it did so, it retained its enzymatic activity. As the aggregation of any enzyme would be expected to lower its activity, we feel it likely that the presence of  $\alpha$ -crystallin prevented both aggregation and inactivation.

#### ACKNOWLEDGEMENTS

This work was funded by a NIH grant EY 087474 to PGF. Thanks to Jodi T. Casselman for excellent technical assistance.

#### REFERENCES

- Bloemendal H, editor. Molecular and cellular biology of the eye lens. New York: Wiley; 1981.
- Maisel H, editor. The Ocular lens: structure, function, and pathology. New York: Dekker; 1985.
- Ingolia TD, Craig EA. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc Natl Acad Sci U S A 1982; 79:2360-4.
- de Jong WW, Leunissen JA, Leenen PJ, Zweers A, Versteeg M. Dogfish alpha-crystallin sequences. Comparison with small heat shock proteins and Schistosoma egg antigen. J Biol Chem 1988; 263:5141-9.
- Merck KB, Groenen PJ, Voorter CE, de Haard-Hoekman WA, Horwitz J, Bloemendal H, de Jong WW. Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of chaperones. J Biol Chem 1993; 268:1046-52.
- Horwitz J. Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci U S A 1992; 89:10449-53.
- Hook DW, Harding JJ. Alpha-crystallin acting as a molecular chaperone protects catalase against steroid-induced inactivation. FEBS Lett 1996; 382:281-4.
- Hook DW, Harding JJ. Molecular chaperones protect catalase against thermal stress. Eur J Biochem 1997; 247:380-5.
- Borkman RF, Knight G, Obi B. The molecular chaperone alpha-crystallin inhibits UV-induced protein aggregation. Exp Eye Res 1996; 62:141-8.
- Ganea E, Harding JJ. Inhibition of 6-phosphogluconate dehydrogenase by carbamylation and protection by alpha-crystallin, a chaperone-like protein. Biochem Biophys Res Commun 1996; 222:626-31.
- Muchowski PJ, Bassuk JA, Lubsen NH, Clark JI. Human alphaB-crystallin. Small heat shock protein and molecular chaperone. J Biol Chem 1997; 272:2578-82.
- Raman B, Ramakrishna T, Rao CM. Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. FEBS Lett 1997; 416:369-72.
- Blakytyn R, Harding JJ. Bovine and human alpha-crystallins as molecular chaperones: prevention of the inactivation of glutathione reductase by fructation. Exp Eye Res 1997; 64:1051-8.
- Marini I, Bucchioni L, Voltarelli M, Del Corso A, Mura U. Alpha-crystallin-like molecular chaperone against the thermal denaturation of lens aldose reductase: the effect of divalent metal ions. Biochem Biophys Res Commun 1995; 212:413-20.
- Carninci P, Nishiyama Y, Westover A, Itoh M, Nagaoka S, Sasaki N, Okazaki Y, Muramatsu M, Hayashizaki Y. Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. Proc Natl Acad Sci U S A 1998; 95:520-4.
- Zietara MS, Skorkowski EF. Thermostability of lactate dehydrogenase LDH-A4 isoenzyme: effect of heat shock protein DnaK on the enzyme activity. Int J Biochem Cell Biol 1995; 27:1169-74.
- Wimmer H, Olsson M, Petersen MT, Hatti-Kaul R, Peterson SB, Muller N. Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. J Biotechnol 1997; 55:85-100.
- Chan HK, Au-Yeung KL, Gonda I. Effects of additives on heat denaturation of rhDNase in solutions. Pharm Res 1996; 13:756-61.
- Tablin F, Oliver AE, Walker NJ, Crowe LM, Crowe JH. Membrane phase transition of intact human platelets: correlation with cold-induced activation. J Cell Physiol 1996; 168:305-13.
- Musch MW, Ciancio MJ, Sarge K, Chang EB. Induction of heat shock protein 70 protects intestinal epithelial IEC-18 cells from oxidant and thermal injury. Am J Physiol 1996; 270(2 Pt 1):C429-36.
- Amin V, Cumming DV, Latchman DS. Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies. Neurosci Lett 1996; 206:45-8.
- Hayashi H, Alia, Mustardy L, Deshniun P, Ida M, Murata N. Transformation of Arabidopsis thaliana with the codA gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 1997; 12:133-42.
- Muchowski PJ, Clark JI. ATP-enhanced molecular chaperone functions of the small heat shock protein human alphaB crystallin. Proc Natl Acad Sci U S A 1998; 95:1004-9.