Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 239-247

PERMANENT SUPPRESSION OF PHASE SEPARATION CATARACT IN CALF LENS USING AMINE MODIFICATION AGENTS

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Received October 10, 1985

Low temperature induced opacification (cold cataract) of the nucleus of young mammalian lenses is associated with a phase separation of proteins in the lens cell cytoplasm. Calf lenses were treated with a variety of imido-esters and N-hydroxysuccinimide-esters, which react specifically with amino groups. Many potent inhibitors of phase separation cataract were identified which lower the opacification temperature by 6°C or more. Lenses generally remain clear, colorless and soft. Furthermore, suppression of the cold cataract temperature is permanent upon removal of excess reagent.

Cold cataract, the opacification of the nucleus of young mammalian lenses induced by low temperatures, is a useful model system for studying pathological cataracts [1-4]. Opacification results from excessive light scattering associated with phase separation of the concentrated protein solution within lens cells. Suppression of the phase separation temperature T_c of calf lens by glycols, aldehydes and acrylamide has been reported [5,6]. In addition, a human cataractous lens partially clarified in 50% glycerol solution [5]. However, the T_c suppression by glycols and acrylamide is reversible when these reagents are diffused out of the lens.

To obtain permanent suppression of T_c we previously used aldehydes to irreversibly crosslink the lens proteins. However, these reagents also

Abbreviations: (MA) methylacetimidate, (IT) imino-thiolane, (DMA) dimethyl-adipimidate, (DMS) dimethylsuberimidate, (DTBP) dimethyl-3,3'-dithiobispropionimidate, (DST) disuccinimidyl tartarate, (DSS) disuccinimidyl suberate, (DTSP) dithiobissuccinimidyl propionate, (EGS) ethylene glycolbissuccinimidyl succinate, (DTSSP) dithiobissulfosuccinimidyl propionate, (BSSS) bissulfosuccinimidyl suberate, (NHS) N-hydroxysuccinimide, (MSH) 2-mercaptoethanol, (DTT) dithiothreitol, (HPLC) high-performance liquid chromatography, (HbS) hemoglobin S

0006-291X/85 $1.50

produce an undesirable hardening and yellowing of the lens [6]. In our search for permanent cold cataract inhibitors we sought reagents which would: a) covalently modify lens proteins, b) link to protein side chain groups suspected to be involved in cataract formation, and c) retain the viscoelastic properties of the lens. Amino groups appear to be implicated in cataractogenesis in the sense that studies of crystallins isolated from cataractous lenses show that their amino groups may be modified through carbamylation [7], nonenzymatic glycosylation [8], steroid binding [9] or isopeptide bond formation [10].

With this in mind we have investigated the effects of imido-esters and NHS-esters on T_c of calf lens. These reagents react covalently and specifically with amines by means of amidination and acylation, respectively. Bifunctional imido- and NHS-esters have been used extensively in crosslinking studies of protein amino groups [11-16]. Moreover, bifunctional modification of amino groups of sickle cell hemoglobin by a bis-imidate [17,18] and bis-salicylates [19] inhibit HbS aggregation and phase separation.

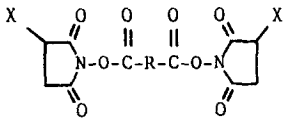
Thus, we have investigated the effects of both mono- and bi-functional reagents [20]. Our present results indicate that many imido- and NHS-esters can dramatically and irreversibly lower T_c of the calf lens while retaining the normal viscoelastic properties of the lens. Although both mono- and bi-functional forms are found to be effective, intermolecular crosslinking of lens crystallins does not appear to be involved in T_c suppression. Independent work [21,22] has recently shown that acetylation of amino groups of bovine and rat crystallins by aspirin, a mono-salicylate, may confer protection against cataract induced by carbamylation.

MATERIALS & METHODS

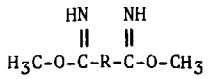
Pairs of eyes from 1-2 week old calves were removed immediately after death and stored on ice several hours before use. Imido-esters and NHS-esters were obtained from Pierce Chemical Co. The general structures and chemical composition of the bifunctional reagents are presented in Table 1. Monofunctional reagents used were methylacetimidate, $CH_3C(=NH)OCH_3$,

TABLE 1: Structures of bifunctional NHS-esters and imido-esters

NHS-ESTERS



IMIDO-ESTERS



reagent	R	X
DSS	$(CH_2)_6$	H
BSSS	$(CH_2)_6$	SO_3^-
DTSP	$(CH_2)_2$-S-S-$(CH_2)_2$	H
DTSSP	$(CH_2)_2$-S-S-$(CH_2)_2$	SO_3^-
DST	$(CHOH)_2$	H
EGS	$(CH_2)_2COO(CH_2)_2OOC(CH_2)_2$	H

reagent	R
DMA	$(CH_2)_4$
DMS	$(CH_2)_6$
DTBP	$(CH_2)_2$-S-S-$(CH_2)_2$

and 2-iminothiolane, $-S(CH_2)_3C(=NH)-$; in addition, the monofunctional forms of DTBP and DTSP were obtained by prior cleavage of the internal -S-S bonds with excess reducing agent, DTT or MSH. Molecular weights of reagents: monofunctional, 100-200, and bifunctional, 250-600. Upon reaction of imido-esters and NHS-esters with protein, the $-C(=NH)R$ and $-C(=O)R$ moieties are covalently linked to protein $-NH_2$ groups, while methanol (CH_3OH) and NHS are the reaction byproducts, respectively.

Solutions of 5 to 50 mM reagent were made up in 5 ml 0.1 M Na-phosphate buffer pH=7.0, I=0.22 (or pH=8.0, I=0.29) just prior to use. While most of the reagents tested were readily soluble in aqueous solutions at room temperature, the NHS-esters EGS, DSS and DTSP dissolved better by heating or by first dissolving in 0.1 ml dimethylsulfoxide and then mixing with 5 ml buffer. A freshly excised lens (average weight 1.0g) was placed in the 5 ml reagent solution in a sealed vial and soaked for 1 to 4 days at room temperature; the contralateral control lens was incubated in buffer. The relative transmittance through the center (nucleus) of both lenses was then measured simultaneously as a function of decreasing temperature; T_c was found when the transmittance decreased to 90% of the maximal value [23]. Control calf lenses opacify between 10-15°C, the variation being mainly attributable to age differences. Hence, it was necessary to use paired lenses, for which T_c differs by less than 1°C. It was found that the T_c of the control lens increases in storage by 1-2°C/day at pH=7.0 and about 5°C/day at pH=8.0; the reason for this phenomenon is not clear.

After incubation, the lenses were rinsed in buffer, decapsulated and homogenized in 5 ml fresh phosphate buffer [24]. The molecular weight distribution of the soluble lens proteins was determined by HPLC analysis on a TSK3000 column [25], and the size distribution of polypeptides by SDS-gel electrophoresis [26].

RESULTS

Fig. 1 is a plot of the relative transmittance vs. temperature for a matched lens pair, one of which was treated with 20 mM DSS, a

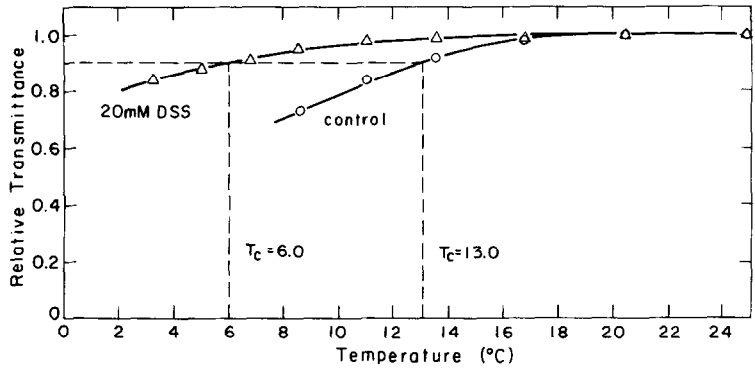


Fig. 1: Temperature dependence of the transparency of calf lenses incubated for 24 hours in (0) 0.1M phosphate buffer pH=7.0, and (Δ) buffer +20mM DSS. Both lenses were subsequently soaked in fresh buffer for an additional 24 hours to remove excess reagent prior to transmittance measurements.

bifunctional NHS-ester, for 24 hours. As seen in Fig. 1, the T_c of the treated lens is 7°C lower than that of the contralateral control lens. Both transmittance curves in Fig. 1 were reversible upon temperature cycling. The photograph in Fig. 2 shows the dramatic improvement of lens clarity at 8°C for the lens treated with 20 mM DSS. Depression of T_c depends both upon time of treatment and the reagent concentration. For example, in Fig. 3 we plot the decrease in T_c as a function of DST concentration from 5-50 mM, after 24 hour treatment.

Representative ΔT_c values for the various reagents acting on calf lenses under physiological conditions of pH (7.0) and ionic strength (0.22)

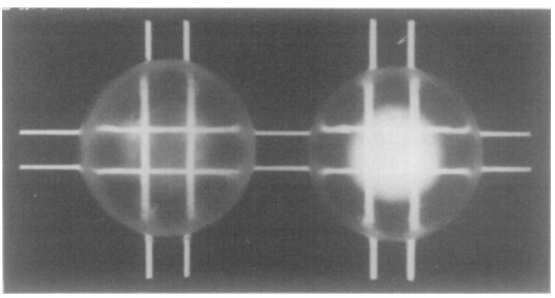


Fig. 2: Example of suppression of cold cataract in calf lens following treatment with 20 mM DSS (left) compared to control (right); photograph taken at 8°C.

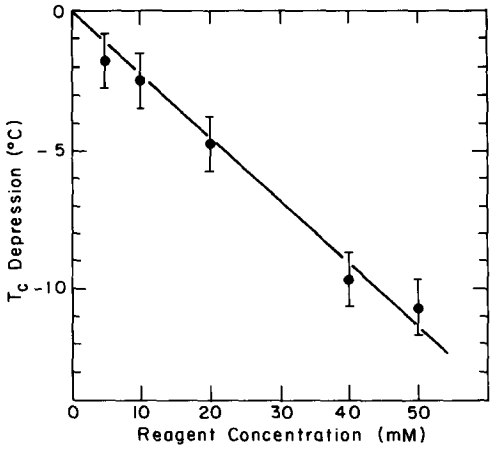


Fig. 3: Depression of T_c in calf lenses as a function of reagent concentration in the incubation mixture. Lens pairs were treated for 24 hours in 0.1M phosphate buffer pH=8.0, with and without the specified concentrations of bifunctional NHS-ester DST.

are summarized in Table 2. It is evident that at concentrations of 20-30 mM many of these reagents can suppress T_c by 6°C or more. Note that the previously used glycols and acrylamide are 10-20 times less effective at these concentrations [5,6]. Some reagents suppress T_c by more than 10°C after only 24 hours incubation; in other words, lens transparency is maintained down to 3°C or less. Lenses generally remain clear, colorless and soft. When treated lenses are returned to buffer solution without reagent, and the excess reagent and reaction byproducts are allowed to

TABLE 2: Effects of NHS-esters and imido-esters on the T_c of calf lens

NHS-ESTERS

Reagent	Conc. (mM)	Time (Hrs)	ΔT_c (°C)
Monofunctional			
DTSP + MSH	20	96	-9
Bifunctional			
DTSP	30	96	-8
DST	20	48	-6
DSS	30	24	>-12
EGS	20	48	-11
BSSS	30	96	-6
DTSSP	30	67	-9

IMIDO-ESTERS

Reagent	Conc. (mM)	Time (Hrs)	ΔT_c (°C)
Monofunctional			
MA	30	96	0
IT	10	24	0
DTBP + DTT	30	24	>-11
Bifunctional			
DMA	30	24	+2
DMS	30	24	-2
DTBP	30	72	-8

diffuse out of lens for 24-48 hours, we observed that 60-70% of the initially observed T_c depression remained. Hence, the effect of these reagents is permanent in the sense that even after removal of the excess modifying reagent, the treated lens opacifies at a lower temperature than the control lens. This is in marked contrast to the reversibility of T_c depression found previously with glycols and acrylamide [6].

All of the NHS-esters tested, both mono-and bi-functional forms, are very potent suppressors of T_c (Table 2, left half). The effectiveness of the monofunctional form of DTSP, produced by cleavage of the internal S-S bond with reducing agent MSH, implies that protein amino group modification alone, without crosslinking, is sufficient to lower T_c. Reducing agents alone such as MSH, DTT or GSH, at concentrations up to 40 mM, had no effect on T_c.

The mono-functional imidoesters MA and IT did not change T_c (Table 2, right half). The only really potent imido-ester was DTBP, both in its mono- and bi-functional form. Most other imido-esters proved to be rather ineffective under these experimental conditions. It should be noted, however, that imido-esters have a pH optimum of 9-10 and that they are rather unstable at physiological pH and susceptible to hydrolysis [26]. Our work in progress indicates that at pH=9 both MA and DMA can suppress T_c of calf lens by an amount comparable to that found with NHS-esters.

While many of these reagents are effective in preventing opacification of the lens nucleus, they generally tend to reduce the clarity of the lens periphery when used at concentrations of 50 mM or more. This is particularly true for the NHS-esters DTSP, DSS and EGS which are less soluble in aqueous solutions; it may be that they begin to precipitate on the lens capsule during incubation. In this respect DTSSP and BSSS, the sulfate-substituted derivatives of DTSP and DSS, are very promising reagents since they are both highly water-soluble and nearly as effective as other NHS-esters in suppressing T_c (Table 2, left half).

After treatment with NHS- or imido-esters, the lenses were extracted and analyzed for protein crosslinking products. In control lenses 98% of total protein was soluble; in treated lenses this decreased only 1% at the most. HPLC analysis indicates that very little change occurs in the size distribution of these soluble crystallins: a slight increase of the β_H-crystallin peak (150,000 M_r) and a broadening of the γ-crystallin peak (20,000 M_r) are characteristically found. SDS-electrophoresis indicates no detectable change in the size distribution of individual polypeptides.

DISCUSSION

Our results clearly indicate that NHS-esters and imido-esters can penetrate through the lens capsule and fiber cell membranes into the cytoplasm of the lens nucleus, to produce a permanent lowering of the phase separation temperature. The mechanism presumably entails covalent modification of the amino groups of the lens proteins. In the case of the NHS-esters the modification leads to loss of the initial positive charges of the protein amino groups. In the case of imido-esters, amidination alone should retain the positive charges, but side reactions of the bound reagent molecules can change the local charge [27,28]. In addition, steric effects associated with bound reagent molecules can play a role in altering the lens protein interactions. In turn, this alteration of the surface properties of the proteins apparently reduces the net attractive interactions between proteins, thereby reducing the phase separation temperature.

Very little, if any, inter-molecular protein crosslinking was evident in lenses treated with bifunctional amine modification reagents. This could be due either to a predominance of intra-molecular crosslinking, to hydrolysis of one end of the reagent, or to the inability of these reagents of 6-16 Å length to span the distance between two protein molecules. No increase of high molecular weight aggregates occurred. Therefore, crosslinking does not seem to be required to depress T_c.

NHS-esters and certain imido-esters are effective in lowering T_c at reagent concentrations at least an order of magnitude less than previously

described for glycols, aldehydes and acrylamide [5,6]. Another important improvement is the fact that permanent suppression of T_c is obtained with very few physical side-effects on the lens. The treated lenses remain soft, clear and colorless. It is a clear advantage of these reagents that they do not affect the visoelastic properties of the lens, which are essential for proper accommodation.

The newly-described amine modification agents have the potential of providing protection against various types of phase separation cataract produced in vivo, and we are presently investigating this hypothesis. Our work in progress has already shown that some of the described amine reagents react more effectively at body temperature; furthermore, they can penetrate the cornea and react effectively with the lens inside the intact enucleated eye. An account of these findings will be published separately.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support provided by the National Eye Institute, NIH (EY05127 and EY05496) and the Jesse B. Cox Charitable Trust. Christophe Coppin acknowledges support from the James Thurber Memorial Fund of Fight for Sight, Inc. New York City for a Student Fellowship (SF85-004).

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